APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ANALYSIS AND PHARMACOKINETICS OF MEPHENOXALONE

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Mephenoxalone is a mild central nervous system depressant with activity resembling that of meprobamate. Since its introduction in 1961 mephenoxalone has been used as an anxiolytic and as a muscle relaxant, although the latter effect is weak.

Preliminary studies on the absorption and disposition of mephenoxalone have been conducted in beagle dogs but no pharmacokinetic data from human studies have been reported, except for a single study in which the biotransformation products present in human urine were identified.

Methods presently available for the determination of mephenoxalone in biological fluids lack the sensitivity, specificity and precision required for detailed pharmacokinetic studies. In this study, a rapid, sensitive, precise reverse-phase high-performance liquid chromatographic method with ultraviolet detection at 200nm was employed for the determination of mephenoxalone in biological fluids. Serum and urine samples were prepared for chromatographic analysis using simple liquid-liquid extraction techniques. The application of the assay to pharmacokinetic studies in humans is presented.

After administration of a single oral dose of 400mg mephenoxalone dispersed in 150ml water to six young, healthy volunteers, the compound was rapidly absorbed with the peak concentration of 8μg/ml occurring after about 1 hour. The elimination half-life was

approximately 3 hours. The drug was extensively metabolized with only about 1 percent of the administered dose being excreted unchanged in the urine after 24 hours.

The bioavailability of a newly developed mephenoxalone-containing tablet was also investigated. The drug was absorbed more rapidly from the tablet than from the dispersed dose. This was attributed to a shorter *in vivo* dissolution time on the basis of *in vitro* tests, but this effect is not expected to be clinically significant.

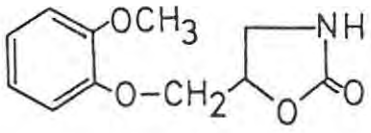
In addition, two human urinary metabolites of mephenoxalone were identified as unconjugated hydroxylated derivatives using thermospray HPLC-mass spectrometry. The plasma protein-binding properties of mephenoxalone were also investigated.

CHAPTER ONE

INTRODUCTION

1.1 PHYSICO-CHEMICAL PROPERTIES OF MEPHENOXALONE

Mephenoxalone (methoxydone, methoxydon, methoxadone, metoxadone) is 5 - (2 - methoxyphenoxymethyl) oxazolidin - 2 - one (1-3). It has the structural formula shown in Fig.1.1. It possesses a cyclic carbamate structure and, as such, differs from meprobamate, the prototype for this series of drugs, which is an open-chain dicarbamate (3,4).



$C_{11}H_{14}NO_4$, mol. mass 223.2

FIGURE 1.1 Mephenoxalone

Mephenoxalone occurs as a tasteless, odourless, white to slightly yellowish-white crystalline powder with a melting point of 143 to 145°C (2). One part of drug is soluble in 100 parts of chloroform, in 110 parts of methylene chloride (dichloromethane), in 170 parts of methanol, in 330 parts of ethanol and in 100 parts of acetone. It is also soluble in propylene glycol but practically insoluble in water and in solvent ether (3, 5).

Although it has been reported that the compound absorbs energy in the ultraviolet (UV) range, with an absorption maximum at 274nm in

chloroformic solution (5), no report exists of a full UV spectrum. A spectrum was therefore determined in this study, the results of which are shown in Fig.2.3. A discussion of the results is presented in Section 2.5.3. Mephenoxalone has also been reported to fluoresce using an excitation wavelength of 300nm and measuring the emitted light at 330nm (uncorrected wavelengths) in ethylene dichloride (6).

The infrared spectrum of mephenoxalone is depicted in Fig.1.2. (7). Absorption at 3250cm^{-1} corresponds to the stretching frequency of the N - H bond. The absorptions at 1720 and 1740cm^{-1} arise from the carbonyl group and at 1510cm^{-1} from the amide functional group.

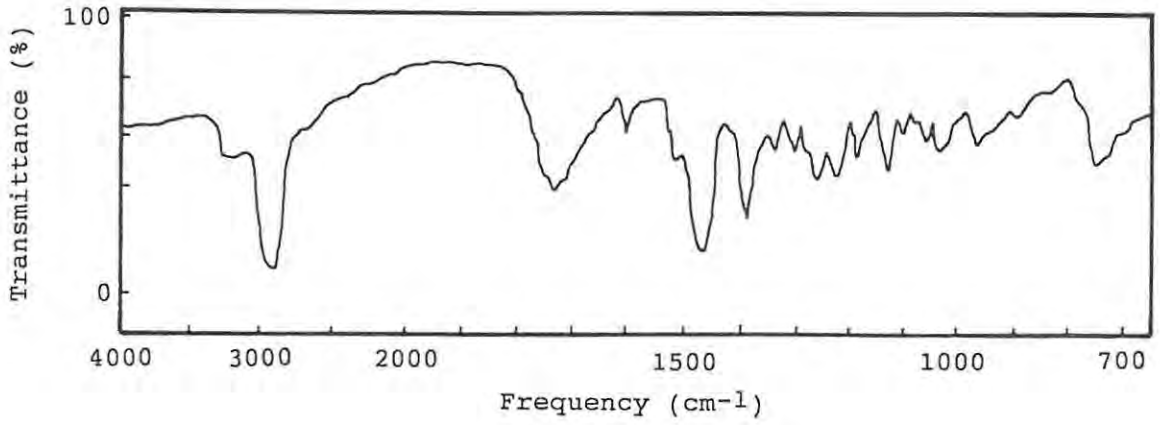


FIGURE 1.2 Infared spectrum of mephenoxalone

Reprinted from Pares *et al.* (7).

1.2 CLINICAL PHARMACOLOGY OF MEPHENOXALONE

1.2.1 Mode of Action and Clinical Uses

Pharmacologically, mephenoxalone resembles meprobamate (2,8-11). Both compounds are classed as central relaxants because they are central nervous system (CNS) depressants with muscle relaxant properties (12). Members of this class may thus be described as mild sedatives with

weak muscle relaxant activities, and their main application is in the management of anxiety and tension states.

Although the action of these agents at a molecular level remains unknown, it is clear that, at a neurophysiological level, reflexes involving interneuronal circuits are depressed (12).

The major sites of the sedative effects of these compounds are the brain stem and subcortical areas. They block the passage of stimuli at the level of the ascending reticular formation (ARF), which is responsible for transmitting and maintaining a state of arousal. The result is a reduced response to sensory stimuli *i.e.* CNS depression (13).

Skeletal muscle relaxation (*i.e.* a reduction in muscle tone) is produced by the blockade of impulses at the interneurons of polysynaptic reflex arcs, mainly at the level of the spinal cord (12,14). This fact is demonstrated by the abolition or diminition of the flexor and crossed extensor reflexes which possess one or more interneurons between the afferent (sensory) and efferent (motor) fibres (12).

The majority of pharmacologic studies of mephenoxalone have been conducted in laboratory animals. Fischer and Szabo (15) and Gray *et al.* (16) have demonstrated that its neuropharmacologic activities are restricted mainly to the supraspinal level. A general sedative action occurs in rats and dogs, the duration of which is approximately four hours following oral administration. A cataleptic action is observed in rats, while the reactions of cats to environmental stimuli are diminished (16). In mice, it modifies seizures resulting from electroshock treatment, reduces motor activity and caffeine hyperactivity, specifically inhibits discriminative avoidance, and mollifies aggressive behaviour induced by electroshock or isolation. Low doses result in reduced rectal temperatures and a significant reduction in the toxicity of *dl*-amphetamine. Other observed neuropharmacologic actions of mephenoxalone include a potentiation of barbiturate narcosis in mice and an enhancement of the analgesic action of morphine in rats. A more recent study by Fischer *et al.*

(17) shows that mephenoxalone increases the analgesic action not only of morphine but also of dihydrocodeine and aminopyrine.

Large doses of the drug injected intraperitoneally into dogs and cats slightly reduce blood pressure (16) but this effect is not observed in rats with stress-induced elevated blood pressure (18). Mephenoxalone has no specific adrenergic or ganglion-blocking actions, but a weak anticholinergic action may be responsible for the reduction in pH and volume of gastric secretions and in gastro-intestinal motility in rats (16). The motility of the bladder and intestine of dogs, however, is unaffected (19).

Clinical studies in man show that mephenoxalone has a tranquilizing action and that it possesses mephenesin-like muscle relaxant properties (16, 20-22). A preliminary investigation using 1000 patients indicated that it had little or no anaesthetic or hypnotic action (20). The use of mephenoxalone (in combination with paracetamol) was investigated in patients with acute or chronic muscular contractions accompanied by pain (22). Good results were obtained in approximately fifty percent of cases. A further study, concerning the effect of the drug in various subchronic and chronic rheumatic diseases, verified the myorelaxant properties of mephenoxalone (21).

Repeated administration of a mephenoxalone-paracetamol combination has been shown to be of value in postoperative pain therapy (23), the pain relief obtained being greater with the combination than with paracetamol alone. Since mephenoxalone has no analgesic activity *per se*, a possible explanation rests on either a delayed cumulative effect or its influence on the perception of pain.

Mephenoxalone is a low potency agent with a moderate dose-response curve and is recommended as being most useful for the treatment of anxiety and tension associated with somatic disorders (16,24). It calms the patient without causing significant drowsiness, thus allowing the person to maintain his usual activity (20). The drug has little or no anaesthetic or hypnotic action and has no therapeutic value in the treatment of psychoses (2,11). Mephenoxalone has a weak

muscle relaxant action which may be useful in the treatment of a variety of conditions where muscle spasm exists *e.g.* torticollis, cervical syndrome, lumbago, myalgia, hernia disci, spasms at bone fractures, arthroses, paraplegia, spondylarthroses, coxarthroses, immobilisations and extensions, and for improvement of flexibility in physiotherapy (2). Skeletal muscle spasm is generally caused by trauma involving muscular overextension or bruising. This results in an acute episode of involuntary muscle contractions. This is often accompanied by pain, interference with function and, occasionally, postural distortion. Treatment may therefore involve muscle relaxant-analgesic combinations. In some cases, tension headaches are responsible for local, sustained muscle contractions which can be treated as above or with muscle relaxant-antianxiety drugs.

1.2.2 Recommended Dosage

Mephenoxalone is administered orally in a tablet dosage form. The recommended adult dose varies from 200 to 400mg, three to four times a day, to a maximum of 2.0g daily. In children (6 to 15 years), the dose is reduced to 100mg three times daily (2).

1.2.3 Adverse Effects and Toxicity

After therapeutic doses of mephenoxalone, the most common adverse drug responses are headache, drowsiness, insomnia, nausea and skin rashes. These effects are generally mild and transient, and occur less frequently than with the prototype, meprobamate (3,9,11).

Although no acute or chronic toxicity has been observed in man, studies on laboratory animals show that serious toxic effects are produced when high doses are used for extended periods. Daily oral doses of 400 to 600mg/kg administered to dogs for 3 to 4 weeks resulted in anorexia, loss of body weight, ataxia, haemolytic anaemia and sequelae (25). However, no toxicity was observed with daily doses of 120mg/kg for a six month period. Acute toxicity studies in mice and rats have shown that oral LD_{50} values for mephenoxalone are greater than 4g/kg body weight (16,26), though it has a greater acute toxicity in newborn rats than in adult rats, the oral LD_{50} values

being approximately 650mg/kg and 3820mg/kg, respectively (26). The increased toxicity in the newborn as compared with adult rats is attributed to their greater sensitivity to CNS depression.

No pathological changes have been observed in the liver, kidneys, adrenal glands or brains of mice, even after chronic intraperitoneal administration (27).

1.2.4 Drug Interactions, Dependence and Precautions

The concomitant administration of other psychotropic drugs (especially phenothiazines or monoamineoxidase inhibitors), which may potentiate the action of mephenoxalone, should be avoided (9,11). It has been reported that mephenoxalone may reduce tolerance to alcohol and although there is no evidence to suggest that it is a dependence-producing substance, the possibility should not be overlooked, especially if the patient has a tendency to abuse drugs (9). For the same reason, the drug should not be discontinued abruptly if the patient has been taking mephenoxalone for a long period of time.

The drug should be discontinued if a skin rash or other hypersensitivity reaction occurs, and patients should be cautioned not to operate a motor vehicle or heavy machinery if they become drowsy or dizzy whilst taking the drug. When mephenoxalone is given in large doses or for extended periods, haemograms and liver function tests should be conducted at regular intervals (9).

1.3 PHARMACOKINETICS OF MEPHENOXALONE

Very little is known about the pharmacokinetics of mephenoxalone in man. The metabolites present in urine have been identified (28) but only one extensive study of the absorption, excretion and metabolic fate of the drug (in beagle dogs) has been reported in the literature (6).

1.3.1. Absorption and Distribution

Morrison (6) showed that in dogs the absorption of mephenoxalone is rapid and almost complete after oral administration. Peak plasma levels of 65 to 68µg/ml were attained 2 to 4 hours after a single oral dose of 100mg/kg, administered in gelatin capsules. An apparent volume of distribution of 110 to 120 percent of body mass was evident. Following oral administration, unchanged mephenoxalone was a major plasma component up to 6 hours after dosing. Since metabolic alteration occurred rapidly after intravenous administration, it is likely that absorption was continuous throughout this interval.

1.3.2 Metabolism

Following oral or intravenous administration, mephenoxalone is almost quantitatively metabolized by dogs to two isomeric phenolic compounds, which are excreted both in the free form and as conjugates with glucuronic acid. Only 1 to 4 percent of the dose is excreted unchanged. The metabolic pathway of mephenoxalone in both cats and rats appears to be identical to the pathway in dogs (6).

The phenolic metabolites are assumed to be the 4'- and 5'-hydroxyl derivatives, formed by hydroxylation *para* to the alkoxyl and methoxyl groups. This pattern of hydroxylation is assumed because although hydroxylation of substituted benzene rings conventionally occurs *ortho* or *para* to groups which are *ortho-para* directing, *e.g.* alkoxyl and methoxyl, the steric effects of these groups on the mephenoxalone molecule are likely to prevent *ortho* substitution (6).

Morrison (6) identified these same phenolic metabolites and their conjugates in the urine of human subjects, the major components comprising the conjugated derivatives. A more recent study conducted by Eckhardt *et al.* (28) demonstrated the presence of ten human urinary metabolites of mephenoxalone. The proposed biotransformation pathway is shown in Fig.1.3. Although some parent drug is excreted unchanged, the majority is degraded by 6 principal routes:

1. The phenoxymethylether bond is cleaved to form *o*-methoxyphenol (Metabolite I) and Metabolite II, the latter being identified by the formation of 3-amino-1,2-propanediol (IIa) after alkaline hydrolysis.
2. Hydroxylation of the benzene ring produces phenolic derivatives (Metabolites IIIa and IIIb).
3. Demethylation to demethylmephenoxalone (Metabolite IV) occurs.
4. Opening of the oxazolidine ring to form 1 - (*o*-methoxyphenoxy)-3-aminopropan-2-ol (Metabolite V).
5. 1-(*o*-Hydroxyphenoxy)-3-aminopropene (Metabolite VI) may be an artefact.
6. Formation of dehydromephenoxalone (Metabolite VII).

Three further unidentified metabolites are present in urine. Metabolites II, IV, V, VI and VII appear to be present as conjugates, and acid hydrolysis or enzymatic cleavage is necessary prior to their isolation and identification.

1.3.3 Elimination

Mephenoxalone is eliminated in a biphasic manner following intravenous administration of the drug to dogs (6). An initial equilibration period of 1 hour is the result of rapid metabolic alteration of the parent compound. The second phase is a first order process in which the metabolic rate is limited by some other process such as the rate of conjugation or excretion of the transformation products. The plasma half-life ($t_{1/2}$) during the latter phase is 2.5 to 3.0 hours. The metabolites are excreted in both urine and faeces after intravenous dosing. The major component found in the faeces is the free form of the less polar of the two phenolic metabolites, while both phenols are excreted, primarily as conjugates, in the urine. Small amounts of the conjugates are, however, also found in the faeces. A possible explanation for the presence of one phenolic metabolite and two conjugated derivatives in the faeces is that conjugates are excreted in the bile and are subsequently almost quantitatively hydrolyzed by glucuronidase in the intestinal tract to form the free phenols, the more polar phenol being reabsorbed into the systemic circulation.

Using either an oral or intravenous administration route in dogs, 80 to 90 percent of the dose is excreted within 24 hours, of which more than 60 percent is excreted in the urine. Repeated oral dosing for four consecutive days, with each dose being larger than the previous one, has shown that the percentage of the dose excreted decreases as the dose increases. This suggests that saturation of a process (*e.g.* rate of conjugation or excretion) has occurred (6).

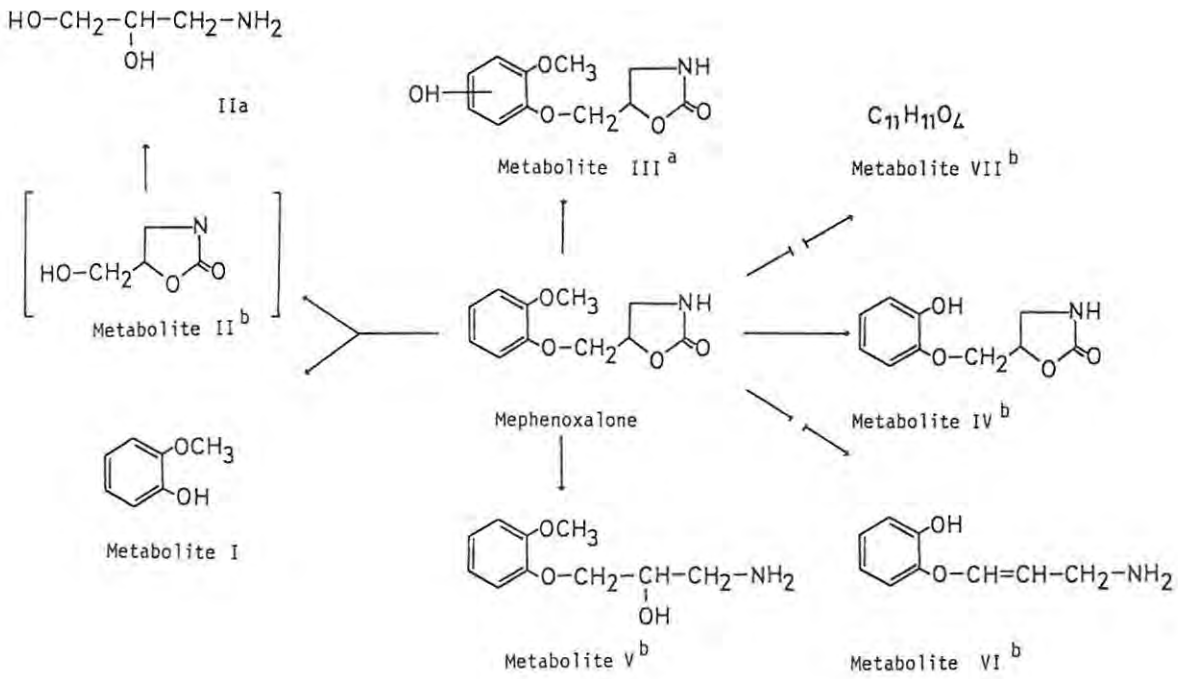


FIGURE 1.3 Biotransformation of mephenoxalone

Adapted from Eckhardt *et al.* (28).

(a) Morrison (6) reported 2 hydroxylated derivatives, Metabolites IIIa and IIIb, which exist as free and conjugated products

(b) exist as conjugates

CHAPTER TWO

DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF MEPHENOXALONE

2.1 INTRODUCTION

High-performance liquid chromatography (HPLC) is a well established technique in pharmaceutical analysis. High efficiency, versatility, selectivity, speed and precision have all contributed to its popularity as a routine analytical tool for drug analysis (29-31).

The majority of HPLC separations are performed on reverse-phase, chemically bonded column packing materials (32). Reverse-phase chromatography (RPC) employs a non-polar stationary phase and a polar mobile phase, thus allowing polar solutes to elute first. This is advantageous for chromatographing biological samples because the hydrophilic endogenous compounds elute first, thereby avoiding the excessively long retention times associated with normal phase packing materials. The microparticulate packing materials also possess the advantages of stability, high efficiency, reproducibility and rapid equilibration. In addition, they allow a broad range of non-polar and polar compounds to be analyzed simultaneously and elution order is often predictable based on the degree of hydrophobicity of each solute (the more hydrophobic the molecule, the greater is the chromatographic retention).

The separation of compounds as they migrate through the column depends on three factors: retention, column efficiency and column selectivity (33, 34). The retention of a compound relies upon the strength of the eluent used, which is usually adjusted to obtain values between 1 and 10 for the column capacity factor, k'. The capacity factor is calculated from Equation 2.1. It may be noted, however, that when the sample contains few components, or when many plates are available, a somewhat lower range (1 to 5 or 0.5 to 2.0) may be preferred (35).

$$k' = \frac{t_r - t_m}{t_m} \qquad \text{Equation 2.1}$$

where t_m = the time for solvent molecules (or an unretained compound) to traverse the column

t_r = the retention time for the compound of interest

The efficiency of the column depends not only upon the size of the packing particles and the quality of the packing material, but also on the packing technique employed, the operating temperature of the column and the eluent flow rate employed. When comparing efficiencies of columns of the same length, the theoretical plate number, N, is a useful parameter. This value is calculated from Equation 2.2 (36).

$$N = 5.54 \left(\frac{t_r}{w_h}\right)^2 \qquad \text{Equation 2.2}$$

where t_r = peak retention

w_h = peak width at half the peak height

Both these values are measured in the same terms of time, volume or distance.

The determination of efficiency is performed with the column placed in-line with the chromatographic system. The efficiency may, therefore, be affected by extracolumn effects such as injection of mass or volume overloads onto the column and detector cell volume effects.

Column selectivity, α, is the ratio of the capacity factors, k'_1 and k'_2, of two compounds, 1 and 2 respectively, and is a function of the stationary phase properties and mobile phase eluting power (33, 37).

The optimization of separation has been covered by many authors (33, 35, 38, 39) and, to a large extent, it can be brought about by changes in mobile phase alone, the stationary phase having been selected in the first place to provide a suitable chromatographic mode for the analysis.

High quality stationary phase packing materials are commercially available. A widely adopted method for column packing is the high pressure slurry technique in which the particles, suspended in a liquid (slurry solvent), are forced into the column by a displacement liquid (packing solvent) under high pressure (40-42). A good slurry solvent is generally found by trial and error but must be capable of removing trapped air from the particle pores and avoiding the formation of agglomerates of packing material. Trapped air is removed by complete wetting of the packing material by the slurry solvent, and the breakdown of agglomerates is encouraged by ultrasonication.

The life of a column depends both on the chemical stability of the packing material and on the physical stability of the packed bed. The chemical stability of silica-based packing materials is primarily affected by mobile phases with pH values below pH 2 and above pH 7 but most drugs can be analyzed within the stable pH range (43). Of more concern are the physical factors which can contribute to a loss of column efficiency during use, including frequent solvent changes, high flow rates and pressures, the injection of air, and the presence of extraneous materials in either the sample or the mobile phase of the HPLC system. Column life, therefore, depends on the analytical conditions employed and the intensity of column use.

Mobile phases employed in RPC with bonded non-polar stationary phases are generally polar solvents or mixtures of polar solvents *e.g.* acetonitrile-water. Mobile phase strengths are usually measured in terms of the solvent polarity and are presented as the elutotropic series. The properties of pure solvents, their sources, and virtues in RPC have been widely published (40, 44). Pre-requisites for an HPLC solvent include high purity, immiscibility with the stationary phase, absence of reactivity with the stationary phase, low boiling point, low viscosity, low cost and good ultraviolet cut-off (if ultraviolet

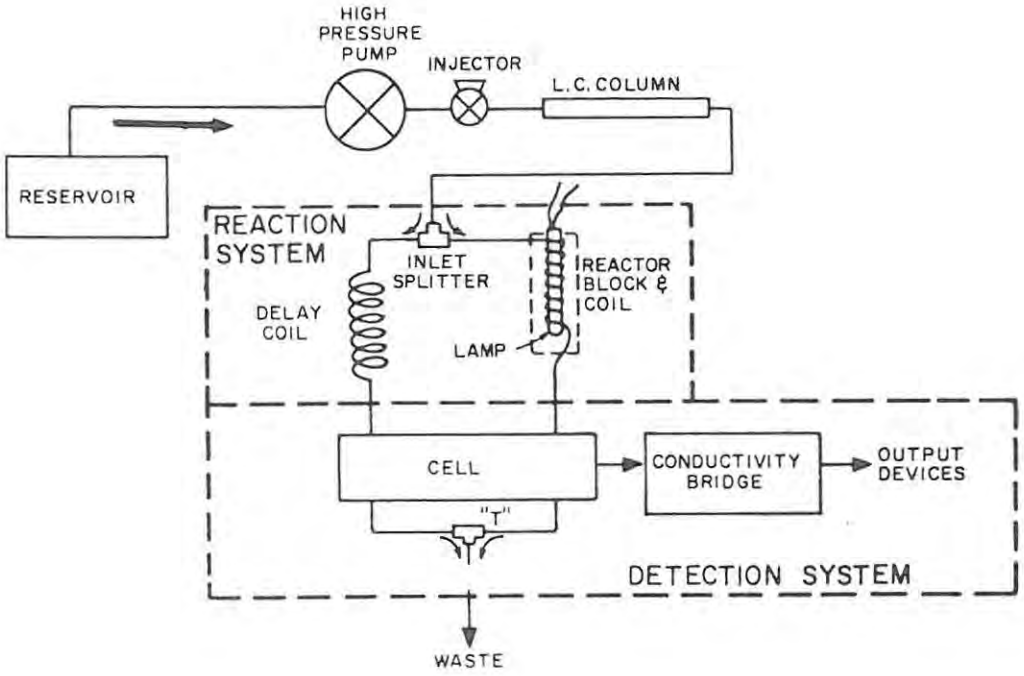
detection is to be employed). Acetonitrile is one of the most popular reverse phase solvents because of its low viscosity, favourable vapour pressure, UV transparency and elution strength. Given a choice between two solvents of similar polarity, the less viscous is always chosen; the lower operating pressures this affords extends column life.

Detection systems employed in HPLC are designed to measure a particular physical or chemical property of a solute in the LC column effluent (45). The fundamental requirements of a detector include a wide linear dynamic range, low drift and noise level, high response, fast response time, ease of operation, and reliability (45, 46). The ultraviolet detector is, at present, the most widely used LC detector because of its high selectivity, reproducibility, low noise characteristics and wide range of application.

A photo-conductivity detector has been used to selectively detect nitrogen-containing compounds (47). This detector measures ionic species in solution and operates either as a selective detector for monitoring photo-reactive compounds or as a general purpose conductivity detector for measuring ionic compounds. When operated in the selective mode, it detects compounds which contain functional groups that are converted to ionic species upon exposure to ultraviolet light. The selectivity of the detector depends on the photo-chemistry of the compounds involved. An overall schematic of a typical photo-conductivity detector is shown in Fig.2.1. In this arrangement the LC column effluent is divided into two equal streams, one leg of which is directed into a UV reactor and then into the analytical side of a differential electrolytic conductivity cell. The other solvent stream passes through a delay coil and into the reference side of the cell. The electrolytic conductivities of the reference and analytical sides of the cell are differentially summed by a conductivity bridge and the resulting output is passed to a recording device. Any compound which is photolyzed in the UV reactor will be detected by this technique. Solvents employed for LC with a photo-conductivity detector must be free from halogens and must be devoid of buffers and salts.

FIGURE 2.1 Photo-conductivity detector

Reproduced from "Photo-conductivity detector operations manual" (47).



Errors incurred in an analytical procedure can be minimized by the use of an internal standard technique in which a known compound is added to the sample solution prior to analysis (48, 49). Errors may be incurred in sample preparation or by variable injection volumes, apparatus and techniques. A basic requirement for an internal standard is that it bears a close structural similarity to the compound being measured. In addition, it must elute near to, and be

completely resolved from, other peaks of interest, it should be added at a concentration which will give a similar peak height to the compound being analyzed, it must not be present in the original sample, and it must behave like the compound of interest during any sample pretreatment. The limitations of the internal standard technique and guidelines for its proper application to the analysis of drugs in biological fluids have been reported (50).

2.2 METHODS AVAILABLE FOR THE ANALYSIS OF MEPHENOXALONE

Few methods have been published in the literature concerning the quantitative analysis of mephenoxalone. The first reported assays were developed by Morrison (6) to analyze mephenoxalone and its metabolites in the biological fluids of laboratory animals. Spectrophotofluorimetric and radiometric procedures were utilized, the former to quantitate parent drug and the latter to measure a total of drug plus metabolites. A liquid-liquid extraction procedure was employed to isolate mephenoxalone from serum and urine, and the ethylene dichloride extracts were measured in a spectrophotofluorimeter by excitation at 300μm and measuring the emitted light at 330μm. This method was reasonably sensitive with a detection limit for mephenoxalone in serum of 0.2μg/ml. Radioactive tracer techniques were employed to determine the total drug plus metabolite concentration at various intervals after dosing with mephenoxalone. Urine samples were counted directly in a liquid scintillation spectrometer, while faecal samples were homogenised in water and combusted (oxygen flask combustion procedure) prior to counting. Blood samples were combusted and plasma samples were either counted directly or combusted first. This technique was time-consuming and the results obtained (total drug plus metabolites concentration) had little significance until combined with those from fluorimetric analysis (parent drug concentration).

An ultraviolet spectrophotometric method has been employed for the determination of mephenoxalone in a tablet dosage form (5). The drug concentration, in chloroformic solution, was measured at a wavelength of 274nm. This method, like spectrophotofluorimetry, has poor selectivity and endogenous interferences from the sample are likely to

occur, particularly when biological fluids are analyzed (48). The sensitivities of these spectrophotometric techniques are typical of direct chemical assay values and are usually not sufficient for measuring the low concentrations found in serum and urine following drug administration.

The most advanced analytical procedure reported to date utilises HPLC to separate mephenoxalone from other sample constituents present in a combination tablet dosage form (2). A reverse phase system with a methanol-water (30/70) mobile phase flowing at 2.0ml/min and a 10µm octadecylsilane (C18) stationary phase gave a retention time of 7.2 minutes. Measurement of mephenoxalone was by ultraviolet detection at 276nm. The limitations of this method centre around the high flow rate used, which, as discussed earlier, produces a high operating pressure and consequently decreases column life and reduces column efficiency. In addition, UV detection at 276nm does not provide adequate sensitivity for measuring the low levels of mephenoxalone found in biological samples.

Reverse-phase HPLC was chosen for this study because of its previous application to the determination of mephenoxalone and its inherent advantages over other analytical methods. A 5µm C18 column was employed for the analysis. A range of slurry solvent-packing solvent combinations were utilized in the column packing procedure and the column life of each packed bed was examined.

Although it is known that mephenoxalone has an absorption maximum at 274nm (5), no record exists of a complete ultraviolet spectrum. The determination of such a spectrum facilitates the choice of a wavelength at which maximum absorption of UV light (λ max) by mephenoxalone occurs, thereby achieving maximum sensitivity. The solubility of mephenoxalone in a range of common laboratory solvents was determined in an attempt to find a suitable solvent, not only for the above-stated determination but also for the routine preparation of stock solutions of the drug.

The application of ultraviolet and photo-conductivity detection methods for the determination of mephenoxalone in human serum and

urine was investigated.

2.3 MATERIALS AND APPARATUS

2.3.1 Materials

The following drugs were used as supplied:

Mephenoxalone (Adcock-Ingram Laboratories Ltd., Johannesburg, South Africa)
Phenacetin (Maybaker (S.A.) (Pty) Ltd., Port Elizabeth, South Africa)
Methocarbamol (Lennon Ltd., Port Elizabeth, South Africa)

All the organic solvents employed in this study were distilled-in-glass grade and were purchased from Burdick and Jackson Laboratories, Muskegon, Mich., USA, with the exception of chloroform which was supplied by Waters Associates, Milford, Mass., USA and 1,2-dichloroethane which was purchased from Riedel-de-Haën, Seelze, W. Germany.

All other reagents were of at least analytical grade quality. Hydrochloric acid and sodium hydroxide were supplied by BDH Chemicals, Poole, England, while phosphoric acid was supplied by Carlo Erba, Milan, Italy.

HPLC-grade water was prepared by reverse osmosis purification followed by passage through a Milli-Q (Millipore Corp., Beford, Mass., USA) system.

2.3.2 High-Performance Liquid Chromatographic System

The HPLC system employed was as follows:

Model M510 liquid chromatographic pump (Waters Associates, Milford, Mass., USA)
Model 710B WISP automated sample injector (Waters Associates, Milford, Mass., USA)
Column packed with 5µm C18 packing material (see Section 2.4.4)

Model LC-UV variable wavelength detector (Pye-Unicam Ltd., Cambridge, England)
Model 561 strip-chart recorder (Perkin Elmer Corp., Norwalk, Conn., USA)

2.3.3 Additional Equipment

The following were employed throughout the study:

Type 2004 MP6 five-figure precision balance (Sartorius BmbH, Göttingen, W. Germany)
Model 601 digital ionalyser (Orion Research Incorp., Cambridge, Mass., USA)
Model 8845-30 ultrasonic cleaner (Cole-Parmer Instrument Co., Chicago, Ill., USA)
N-Evap Model 112 analytical evaporator (Organomation Associates Inc., South Berlin, Mass., USA)
Model HN-SII general purpose centrifuge (Internation Equipment Co., Needham Heights, Mass., USA)
Model M-16710-12 'maxi-mix' vortex mixer (Thermolyne Corp., Dubuque, Iowa, USA)
Model 3521 reciprocating mechanical shaker (Lab-Line Instruments, Inc., Melrose Park, Ill., USA)
Fixed and variable volume Transferpettes (Brand, W. Germany),
Magnetic stirrer (Gallenkamp, England)

Grade A glassware was employed throughout the study. Before use, all glassware was cleaned in Decon 75 concentrate (Atomic Export Import Corp. (Pty) Ltd., Johannesburg, South Africa), thoroughly rinsed with distilled water and HPLC-grade water, and then dried.

2.4 EXPERIMENTAL

2.4.1 Standard Solutions

Each stock solution was prepared by dissolving 25mg of the appropriate drug sample in acetonitrile and making up to volume with acetonitrile in a 25ml volumetric flask. Dilutions were made, with either water or mobile phase, to produce the required concentrations.

A standard C18 column test mixture was prepared by mixing 10µg each of benzamide, benzophenone and biphenyl with 0.5ml of a 1 in 50 dilution of benzene in acetonitrile in a 20ml volumetric flask and making up to volume with acetonitrile.

2.4.2 Chromatographic Conditions

The following chromatographic conditions were utilized:

HPLC system	:	see Section 2.3.2.
UV detection wavelength	:	200nm
Sensitivity	:	0.16 AUFS back-off (coarse) 5 recorder input 10mV
Chartspeed	:	5mm/min
Flow rate	:	1.0ml/min
Pressure	:	3000 psi
Temperature	:	ambient
Mobile phase	:	acetonitrile-water (35/65)

2.4.3 Mobile Phase Preparation

All mobile phases used in this study were prepared without correcting for the volume changes that occur upon mixing of the aqueous and organic components. The solvent mixtures were degassed and filtered through a 0.45µm membrane filter (Type HVLP, Millipore Corp., Bedford,

Mass., USA) prior to use. All mixtures were freshly prepared on the day of analysis.

The phosphate buffers were prepared by transferring a specific volume of phosphoric acid (depending upon the required molarity) to a 1 litre volumetric flask and making up to volume with HPLC-grade water. Sodium hydroxide pellets were then added to the acid/water mixture, with constant stirring on a magnetic stirrer, until the desired pH was attained.

2.4.4 Columns and Column Packing Procedure

Empty steel columns, 25cm x 3.9mm i.d. were packed with Techsil 5µm octadecylsilane (C18) material (HPLC Technology Ltd., Cheshire, England) by a high pressure slurry technique using an HPLC column packer (HPLC Technology Ltd., Cheshire, England). The following slurry solvent - displacement solvent combinations, respectively, were used to pack four different columns of the packing material:

1. methanol - isopropanol
2. methanol - carbon tetrachloride
3. carbon tetrachloride - carbon tetrachloride
4. carbon tetrachloride - methanol

Packing Procedure

Removal of old packing contents was followed by thorough rinsing of the empty steel column with methanol, after which the tubing was dried. The column end-fittings and frits were sonicated in nitric acid and rinsed with methanol. For every column packed, three grams of packing material was slurried in 30ml of slurry solvent and then sonicated for one minute. The slurry was transferred without delay into the column packing reservoir and slurry solvent added to fill the reservoir. The packing process was then started. One hundred and twenty millilitres of degassed and filtered displacement solvent was allowed to pump through the column before the assembly was inverted, without interruption of flow, and a further 120ml pumped through the column. The pump was then depressurised and, after a half hour

stabilization period, the column was disconnected. Any residual material at the top of the column was carefully removed before placing the frit and end fitting onto the column. Finally, the packed column was washed with 30ml of methanol, followed by 50ml of acetonitrile-water (75/25), and then tested, as described below.

2.4.5. Column Performance Testing

The following chromatographic conditions were employed:

HPLC system	:	see Section 2.3.2
UV detection wavelength	:	254nm
Sensitivity	:	0.04 AUFS back-off (coarse) 5 recorder input 10mV
Chartspeed	:	1cm/min
Temperature	:	ambient
Mobile phase	:	acetonitrile-water (75/25)
Flow rate	:	1.0ml/min

Aliquots of 5µl of a 1 in 50 dilution of standard C18 column test mixture (section 2.4.1) were injected into the HPLC system. The number of theoretical plates for each column was calculated (Equation 2.2). Column performance was measured again at the end of the column life and factors which contributed to any drop in efficiency were noted. The effectiveness of repairing a top-end column void by the application of a paste of Techsil 5µm C18 material in methanol was examined.

2.4.6 Mephenoxalone Solubility Study

The solubility of mephenoxalone in each of the solvents listed below was determined by the progressive addition of solvent to 25.0mg samples of the drug. After each solvent addition, the sample was sonicated for one minute and dissolution was ascertained as being complete when particulate matter was no longer visible.

The following solvents were tested:
Water, acetonitrile, methanol, chloroform, methylene chloride (dichloromethane), ethyl acetate, diethyl ether, heptane, benzene, carbon tetrachloride, isopropanol, amyl alcohol, hydrochloric acid (0.1N), sodium hydroxide (0.1N).

2.4.7 Mephenoxalone Ultraviolet Spectrum

The UV spectrum of mephenoxalone was determined using a scanning spectrophotometer (Model Acta MVI, Beckman Instruments, Inc., San Ramon, California). A 10µg/ml solution of the drug in acetonitrile was transferred to a standard 1cm pathlength cuvette while pure solvent was placed in the reference cuvette. A plot of absorbance *versus* wavelength was automatically generated under the following conditions:

Sensitivity	:	1
Scan speed	:	1/4nm/second
Upper wavelength	:	310nm
Lower wavelength	:	200nm
Mode selector	:	DB Servo slit position
Slit program selector	:	Program mode

An ultraviolet spectrum of mephenoxalone in methanol was determined by the same procedure.

2.4.8 Detectors

2.4.8.1 Ultraviolet Detector

Aliquots of 10µl of a 1.5µg/ml solution of mephenoxalone in mobile phase were injected into the liquid chromatographic system under the conditions previously described (Section 2.4.2). Samples were then re-injected using detector wavelengths of 221nm and 276nm. The limit of detection for the drug at 200nm was ascertained by injecting 10µl samples of serially diluted mephenoxalone solutions until a signal-to-noise (S/N) ratio of 3:1 was obtained.

2.4.8.2 Photo-conductivity Detector

The use of a photo-conductivity detector (Model 965, Tracor, Inc., Austin, Texas) was also investigated. The following detector operating conditions were utilized:

Mode	:	Opr Fltr
UV Source	:	Zinc lamp
Sensitivity range	:	1
Output attenuation	:	10

On-column loads of 1.0µg of mephenoxalone in 10µl of acetonitrile were injected into the chromatographic system using a mobile phase of acetonitrile-water at a flow rate of 1.0ml/min. The composition of the mobile phase was varied from a 40/60 to a 30/70 ratio of organic/aqueous components. Using a 30/70 mobile phase, the flow rate was then reduced to 0.8ml/min in an attempt to enhance sensitivity by increasing the residence period of the sample in the UV reaction system.

2.4.9 Mobile Phase Development

The studies performed in this section pertain to optimization of the mobile phase composition with respect to ultraviolet detection. Preliminary studies were performed with a methanol-water (30/70) mobile phase. The organic component was then substituted with acetonitrile, and numerous mobile phases, differing only in organic/aqueous ratios, were tested for their ability to resolve mephenoxalone from the two internal standards, methocarbamol and phenacetin. The effect of varying the flow rate, between 0.4ml/min and 1.2ml/min, on the retention characteristics and capacity factors of these compounds was investigated.

The effect of added buffer, buffer strength and pH on the elution patterns of these compounds was also examined. A mobile phase of 35/65 acetonitrile-phosphate buffer (0.005 - 0.1moles/litre; pH 2 - 7) at a flow rate of 1.0ml/min was used for this purpose.

2.4.10 Internal Standards

The following drug standards were tested as possible candidates for use as an internal standard : phenacetin, methocarbamol, meprobamate, phenytoin, hexoprenaline hydrochloride, caffeine and phenobarbitone. Stock solutions containing 1mg/ml in appropriate solvents were freshly prepared and then diluted 1 in 20. Ten microlitre aliquots were then injected into the HPLC system under the conditions previously described (Section 2.4.2).

2.5 RESULTS AND DISCUSSION

2.5.1 Column

2.5.1.1 Column Performance and Stability

The number of theoretical plates for each packed column, calculated from Equation 2.2 using the third eluting peak (benzophenone) of the standard test mixture, is shown in Table 2.1. The plate number at the end of the column life is also shown. The end of column life was ascertained as being the point at which a loss of resolution occurred between mephenoxalone and internal standard (phenacetin) as a result of the overlap of peaks or pronounced peak tailing.

TABLE 2.1 Efficiencies of packed 5µm C18 columns

SLURRY SOLVENT	PACKING SOLVENT	INITIAL PLATE NUMBER (a)	PLATE NUMBER AT END OF COLUMN LIFE
Methanol	Isopropanol	1000	700
Methanol	Carbon tetrachloride	2000	1000
Carbon tetrachloride	Carbon tetrachloride	>6000	1000
Carbon tetrachloride	Methanol	>6000	1000

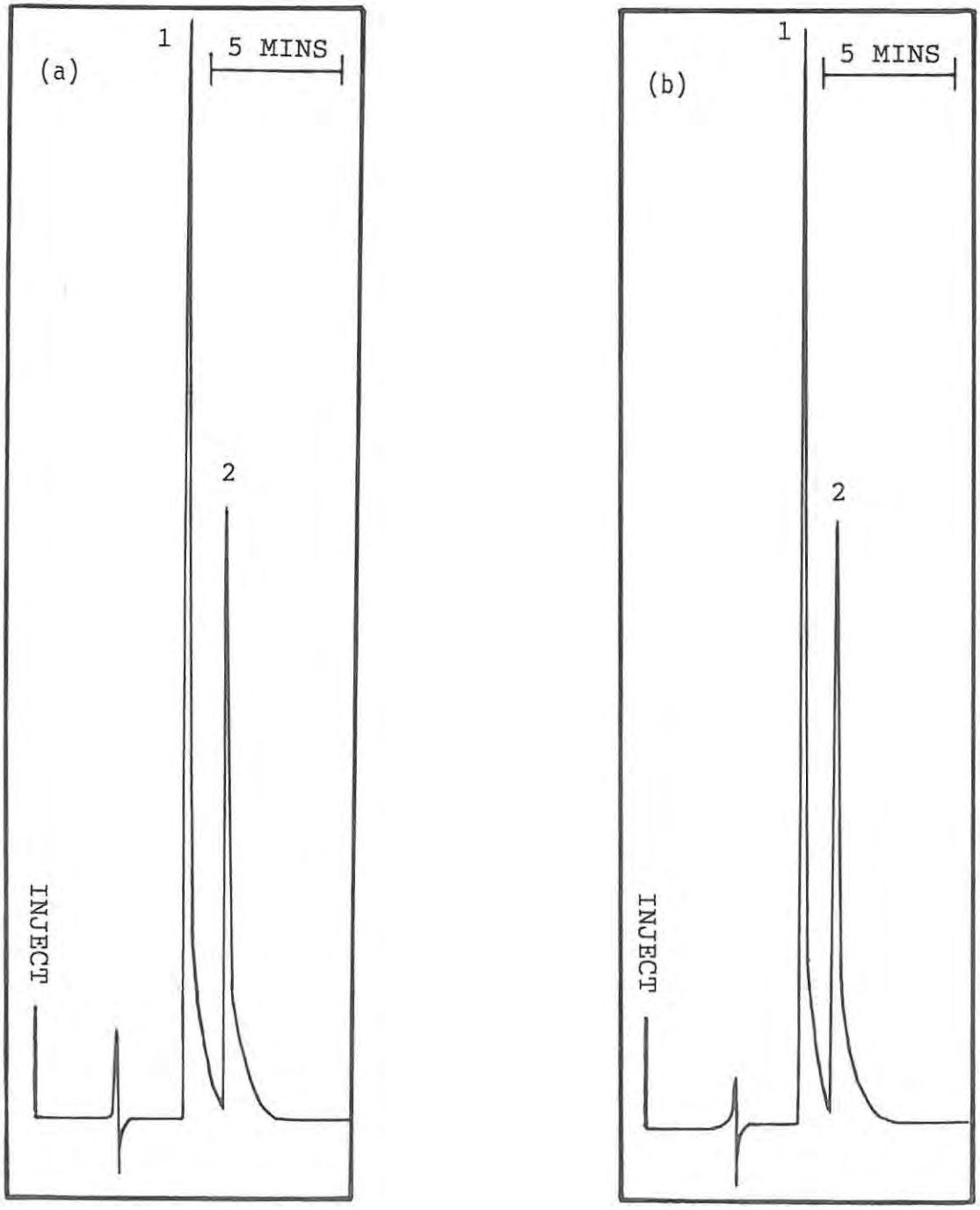
(a) 25cm column

When carbon tetrachloride was employed as the slurry solvent with either methanol or carbon tetrachloride as the displacement solvent, superior columns (in terms of efficiency) were produced by comparison to columns packed with the alternative solvent combinations (Section 2.4.4). Carbon tetrachloride appeared to completely wet the packing materials whereas the other slurry solvents did not. It has been reported that the addition of small amounts of methanol to a carbon tetrachloride slurry of 5μm C8 packings produces columns with higher plate counts than those slurried in carbon tetrachloride alone (5) but this effect was not investigated in this study.

A dramatic reduction in the performance of the column packed using a methanol slurry solvent and an isopropanol packing solvent occurred over a period of one month of continuous use. The short column life may have been the result of poor wetting properties of the slurry solvent but it was more likely the result of frequent changes in mobile phase composition during the method development stages or the high operating pressures (3000 to 3500 psi) of the column. The chromatograms produced at the end of the column life showed pronounced peak tailing and loss of resolution between mephenoxalone and the internal standard, phenacetin, as depicted in Fig.2.2(a). The top of the column was opened and a void was observed. An attempt was made to rejuvenate column performance by filling the void with a methanolic paste of 5μm C18 packing material but this did not produce any improvement in peak shapes or resolution between the two compounds (Fig.2.2(b)).

The column packed with methanol as the slurry solvent and carbon tetrachloride as the displacement solvent also had a very short life. This column was employed for the initial stages of the development of an extraction method to isolate mephenoxalone from serum and urine samples. A guard column was not, however, used prior to the main column and contaminants from the injected samples thus built up at the top of the column, causing excessive back-pressure. Cleaning the column frits by sonication in nitric acid and rinsing with methanol reduced the pressure on the column but a void had already formed at the top of the column. As before, no improvement in resolution between mephenoxalone and phenacetin was obtained by application of a

FIGURE 2.2 Chromatograms showing 200ng on-column loads of mephenoxalone[1] and internal standard, phenacetin[2] (a) before application and (b) after application of a paste of packing material (chromatographic conditions as in Section 2.4.2, 0.08AUFS)



paste of packing material.

When carbon tetrachloride was employed as both the slurry solvent and the packing solvent, a stable column was produced. No loss of performance was observed until buffered mobile phases were utilized; after using solvents varying over a wide pH range (2.5 to 7.0), a thirty percent drop in effiency was observed. The high operating pressures (3000 psi) also contributed to a loss of performance over a long period of time. The accidental injection of air in nearly fifty samples caused a thirty percent drop in plate number and eventually destroyed the column.

The column packed with carbon tetrachloride as the slurry solvent and methanol as the displacement solvent was employed for validation of the method developed for measuring mephenoxalone in biological fluids and also for analyzing the samples from the first set of clinical trials. The gradual build up of pressure, resulting in compaction of the column bed, was responsible for reducing column performance. The column life, however, exceeded six months, which was regarded as reasonable considering the intensity of use. The advantage of using methanol instead of carbon tetrachloride as the displacement solvent is that the former is compatible with the HPLC packing apparatus whereas the latter tends to be corrosive due to the formation of HCl.

2.5.1.2 Choice of Column

A 10µm C18 column has been previously applied to the determination of mephenoxalone (2). However, 5µm packing material offers improved efficiency compared with 10µm materials and a 5µm C18 column was therefore adopted for this study. The most suitable packing procedure was that using methanol as the packing solvent and carbon tetrachloride as the slurry solvent; a high efficiency column with a relatively long analytical life was thus produced.

2.5.2. Mephenoxalone Solubility

The solubility of mephenoxalone in a range of solvents is shown in Table 2.2. The values obtained for methanol, methylene chloride,

diethyl ether and water compare favourably with reported values (2, 5). Of the solvents tested, acetonitrile and methylene chloride were the best, requiring 30 to 100ml of solvent to dissolve 1mg of solute.

TABLE 2.2 Solubility of mephenoxalone

SOLVENT	MILLILITRES OF SOLVENT REQUIRED TO DISSOLVE ONE MILLIGRAM OF SOLUTE	SOLUBILITY TERM[a]
Acetonitrile Methylene chloride	30 to 100	Sparingly soluble
Methanol Chloroform Isopropanol Amyl alcohol Ethyl acetate	100 to 1000	Slightly soluble
Benzene Carbon tetrachloride	1000 to 10 000	Very slightly soluble
Heptane Diethyl ether Water 0.1N hydrochloric acid 0.1N sodium hydroxide	10 000 and over	Practically insoluble or insoluble

[a] according to the USP XX

2.5.3 Mephenoxalone Ultraviolet Spectrum

For the purpose of recording an ultraviolet spectrum of mephenoxalone over the range 200-310nm, a solvent with a sufficiently low UV cut-off wavelength must be used. Acetonitrile, heptane, methanol, isopropanol and water have cut-off values below 202nm (44, 45) but only the first two have favourable properties as solvents for dissolving mephenoxalone at the required concentrations.

The ultraviolet spectrum of mephenoxalone in acetonitrile and in methanol is shown in Fig.2.3. The compound has three absorption maxima in the wavelength range examined (190-310nm). The maxima are 276nm, 220nm and 200nm, the latter being the wavelength of maximum UV absorption for the drug. These maxima are in agreement with predictions made according to the chemical structure of the compound : alkyl and *o*-alkyl substituted benzene derivatives containing not more than three substituents exhibit characteristic UV absorption maxima at 226 $\pm$ 6nm and 273 $\pm$ 7nm in aqueous acid (52). Mephenoxalone, as an *o*-alkyl substituted benzene derivative, was therefore expected to display these maxima.

2.5.4 Detectors

2.5.4.1 Ultraviolet Detection

The LC detector response at wavelengths corresponding to the three absorption maxima of mephenoxalone are shown in Fig.2.4. The chromatograms were generated under identical conditions, except for the change in wavelength. The greatest sensitivity, in terms of detector response, was obtained at 200nm. The noise level is lower at the longer wavelengths (220nm, 276nm) but the sensitivity is poor. The limit of detection at 200nm was a 2ng on-column load of mephenoxalone (Fig.2.5). The detector response was reproducible and stable to ambient temperature fluctuations.

FIGURE 2.3 Ultraviolet spectrum of mephenoxalone in (1) acetonitrile and (2) methanol

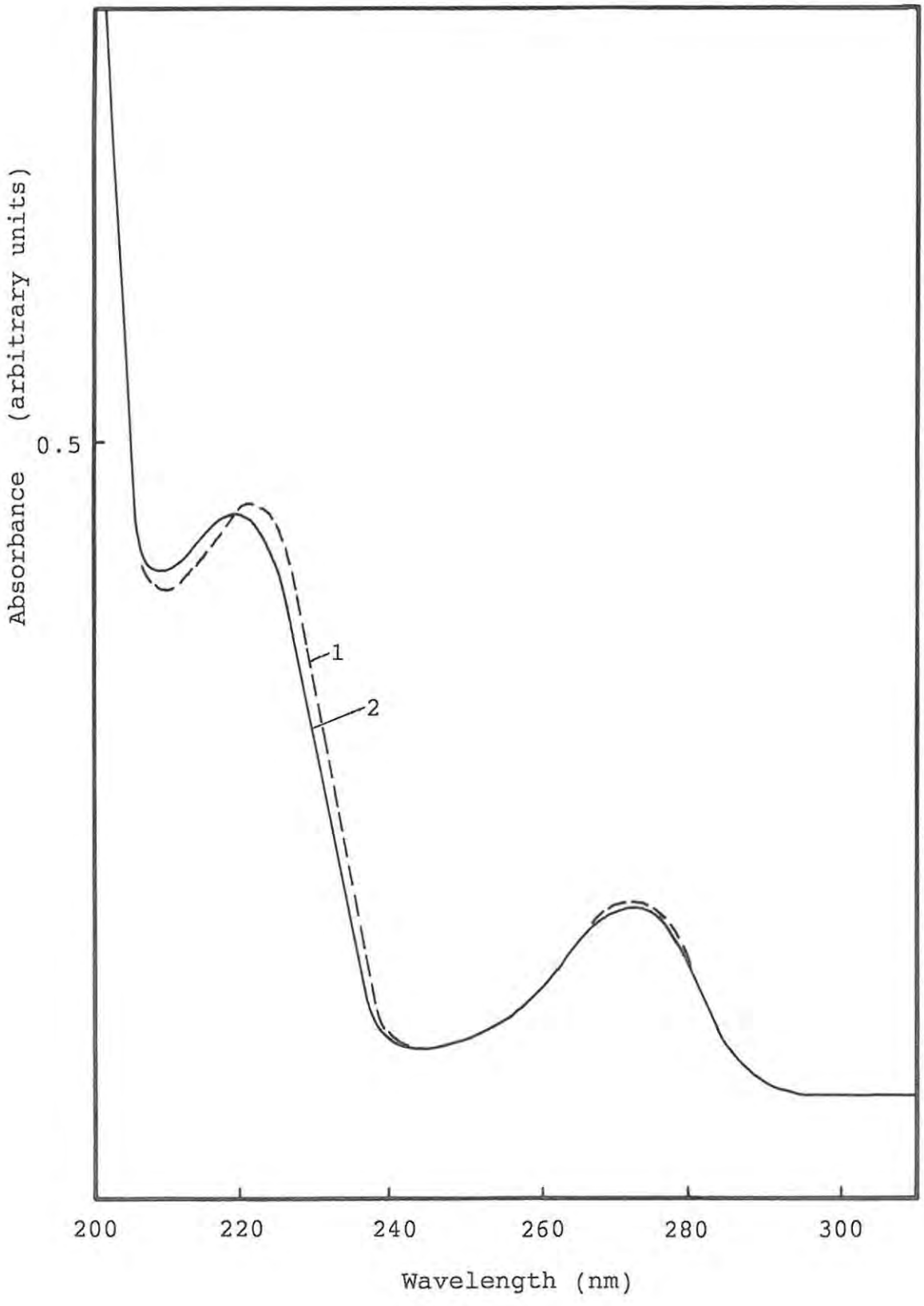


FIGURE 2.4 Chromatograms showing detector response to a 15ng on-column load of mephenoxalone[1] at detector wavelengths of (a) 200nm (b) 220nm and (c) 276nm (chromatographic conditions as in Section 2.4.2)

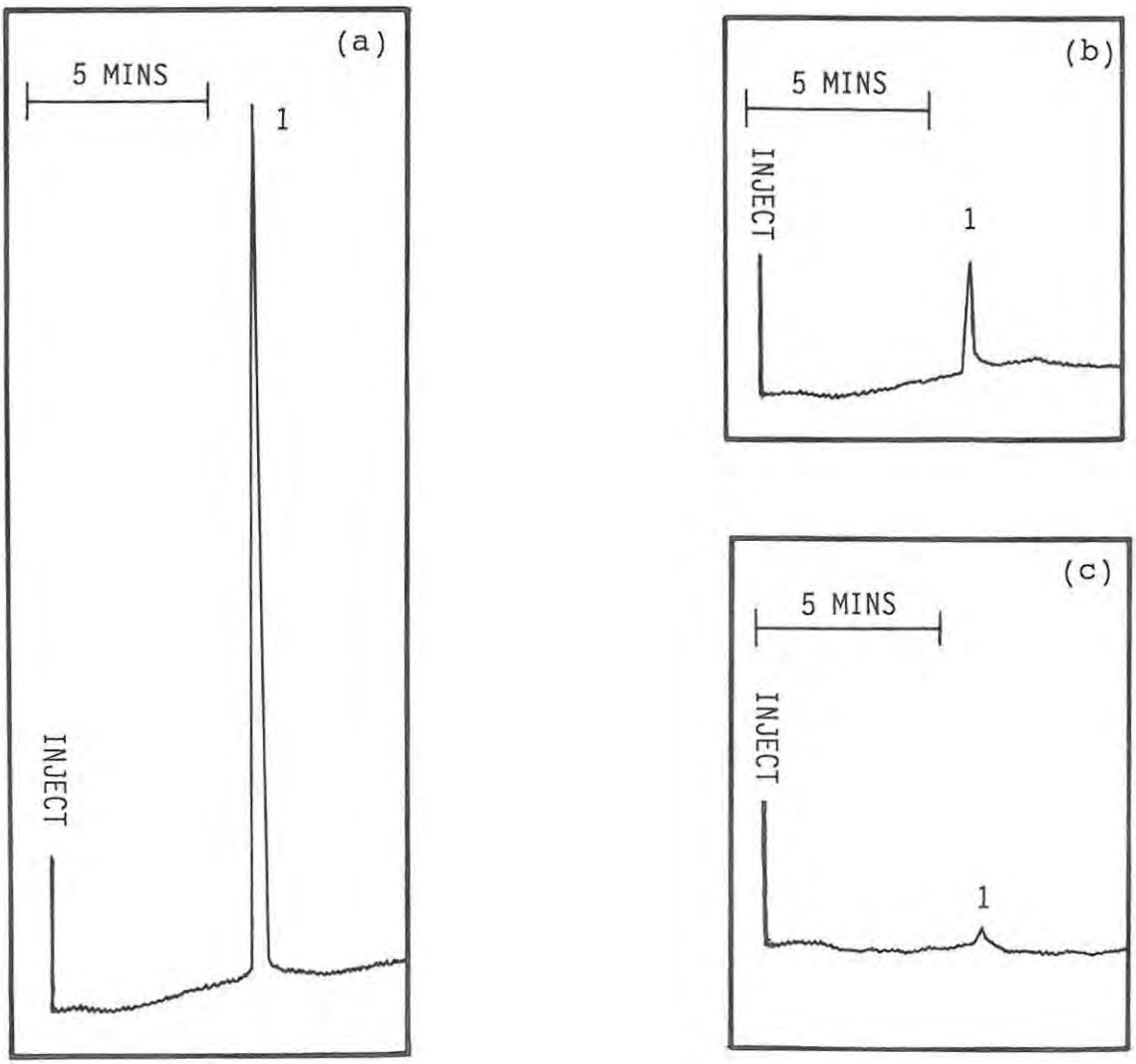
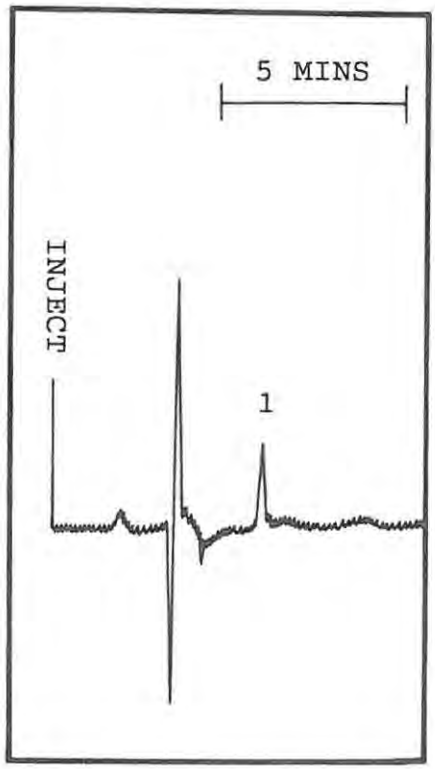


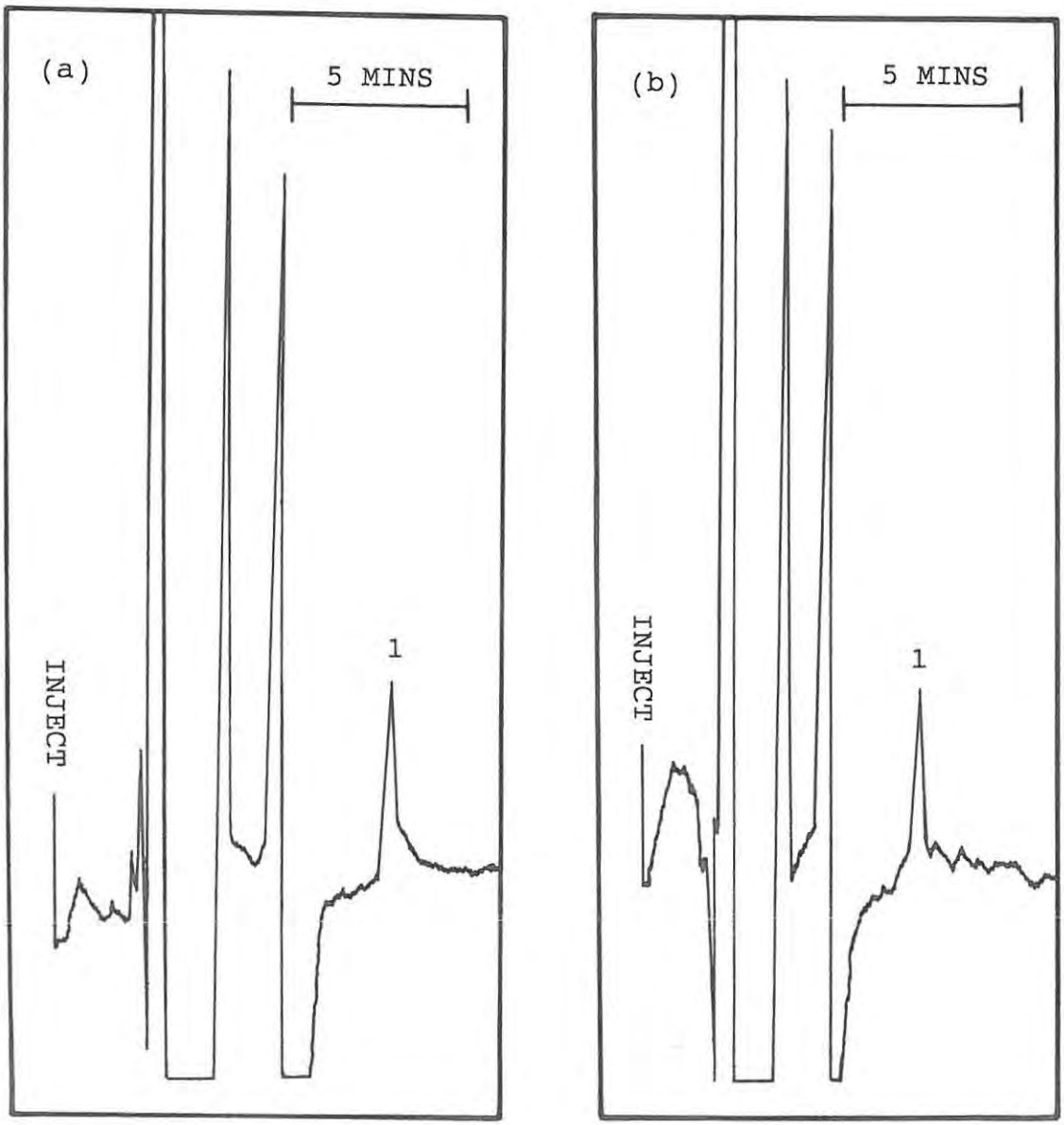
FIGURE 2.5 Chromatogram showing the limit of detection for mephenoxalone at 200nm (on-column load = 2ng: chromatographic conditions as in Section 2.4.8.1)



2.5.4.2 Photo-conductivity Detection

Mephenoxalone appears to undergo weak ionization under the conditions employed (Section 2.4.8.2). A mobile phase of acetonitrile-water 30/70 at 1.0ml/min resolved mephenoxalone and the large solvent peaks, and a minimum detectable limit of 500ng was obtained herewith. The chromatograms generated using flow rates of 0.8ml/min and 1.0ml/min are depicted in Fig.2.6. Theoretically, the longer the time a compound is exposed to UV light in the reaction system of the

FIGURE 2.6 Chromatograms showing photo-conductivity detection of mephenoxalone[1] at (a) 0.8ml/min (b) 1.0ml/min (on-column load = 1 µg; chromatographic conditions as in Section 2.4.8.2)



detector, the more sample ionization is obtained. The results of this study however, show that there was no significant increase in the sensitivity of the analysis at the lower flow rate (0.8ml/min), although the short-term baseline noise and drift were less than at the higher flow rate (1.0ml/min).

2.5.4.3 Choice of Detector

A sensitive mode of detection is required for the determination of mephenoxalone in biological fluids. Photo-conductivity detection lacked the required sensitivity. Ultraviolet detection, on the other provided an adequate detection sensitivity for monitoring realistic drug concentrations. In addition, UV detection was a more convenient and a more reproducible means of monitoring the LC column effluent and was, therefore, adopted for all further studies.

2.5.5 Choice of Mobile Phase

The choice of mobile phase is governed by the need for adequate retention of mephenoxalone on the column, resolution from the internal standard, good peak shape, and a reasonable analysis time and operating pressure. If the method is to be applied to biological fluids, a retention time of at least 4 minutes is necessary to allow for the prior clearance of endogenous compounds from the column. The use of ultraviolet detection at 200nm limits the choice of mobile phase constituents to those with UV cut-off values below 200nm (44). Methanol, despite having a UV cut-off at 205nm, can be used in small amounts. Acetonitrile (UV cut-off at 200nm) has supplanted methanol as the most widely used reverse-phase organic modifier because of its numerous favourable properties, as discussed earlier.

A methanol-water (30/70) mobile phase has been previously employed for the HPLC analysis of mephenoxalone (2). This mixture, however, has a high viscosity (40) and the maximum flow rate which can be used is limited by the back-pressure generated. A maximum flow rate of 1.0ml/min at an operating pressure of 4000psi could be used in this laboratory. However, under these conditions mephenoxalone was retained on the column for more than 14 minutes and a peak with poor

symmetry and shape was obtained in the chromatogram. Substitution of methanol with acetonitrile, in the same proportion (30/70) and at the same flow rate (1.0ml/min), provided greatly improved results but the column pressure (3600psi) was still considered to be excessive for routine analysis. Since mobile phase viscosity is reduced as the proportion of acetonitrile is increased (40), an organic-aqueous (35/65) solvent mixture, flowing at 1.0ml/min, was tested. Using this mobile phase, a good compromise was achieved between analysis time, resolution from internal standards, and column pressure. Under these conditions, the retention times of mephenoxalone and the internal standards, methocarbamol and phenacetin, were 5.2, 4.0 and 6.5 minutes, respectively, as shown in Fig.2.7. It is well known that the efficiency of a column is optimum at a particular flow rate (40). For a 5µm C18 column, this maximum value lies closest to 1.0ml/min.

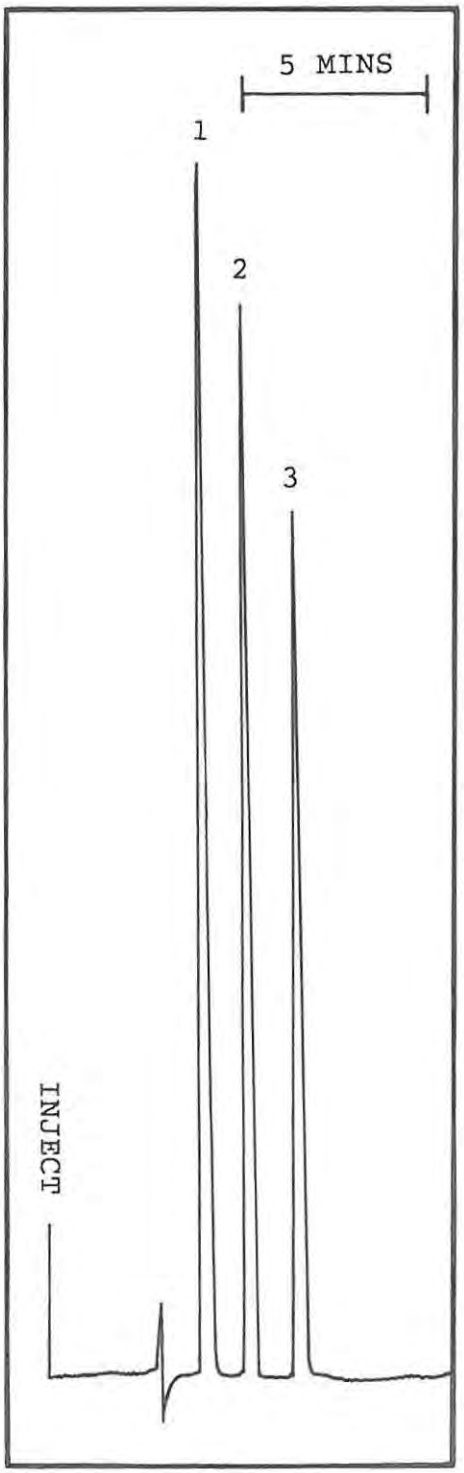
An investigation of the effect of adding buffers to the mobile phase on the retention characteristics of mephenoxalone, phenacetin and methocarbamol revealed that the capacity factors of these compounds did not change with variations in either pH (2 - 7) or phosphoric acid molarity (0.005 - 0.1 moles/litre). This indicates these drug molecules do not possess any significantly ionizable functional groups.

2.5.6 Choice of Internal Standard

Of all the compounds tested, only methocarbamol and phenacetin were suitable candidates for use as internal standards in the analysis of mephenoxalone. Both substances are structurally similar to the compound of interest (Fig.2.8), both have comparable extraction ratios to that of mephenoxalone and both are readily detectable at 200nm. Using the HPLC conditions described in Section 2.4.2, these compounds were resolved from the analyte substance, as shown in Fig.2.7. Methocarbamol eluted prior to mephenoxalone and co-eluted with a mephenoxalone metabolite present in extracted biological fluids. This internal standard is, therefore, suitable only for aqueous studies.

In addition to the fact that phenacetin has a greater retention time than mephenoxalone, it is no longer used clinically and it is,

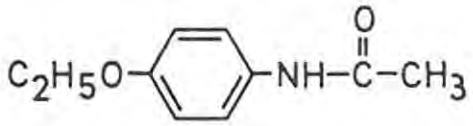
FIGURE 2.7 Chromatogram showing elution profile of methocarbamol[1], mephenoxalone[2], and phenacetin[3], using a mobile phase of acetonitrile-water (35/65) at 1.0ml/min (chromatographic conditions as in Section 2.4.2; injection volume 10µl)



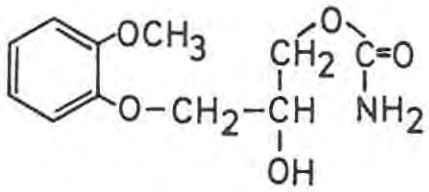
therefore, the internal standard of choice for the determination of mephenoxalone in serum and urine.

FIGURE 2.8 Structures of (1) phenacetin and (2) methocarbamol

(1)



(2)



2.6 CONCLUSIONS

An HPLC method using a Techsil 5µm C18 stationary phase and an acetonitrile-water (35/65) mobile phase was suitable for the analysis of mephenoxalone in aqueous samples and in biological fluids. A column packed using a slurry solvent of carbon tetrachloride and a displacement solvent of methanol provided excellent effienciency, stability and column life. The column was operated at ambient temperature and at an optimum flow rate of 1.0ml/min. Methocarbamol was a suitable internal standard for aqueous studies while phenacetin proved to be the compound of choice for the analysis of biological fluids. Ultraviolet detection at 200nm provided adequate sensitivity for the determination of mephenoxalone in serum and urine following oral administration of the drug. The use of a photo-conductivity detector for measuring mephenoxalone was excluded from further investigation because of its low sensitivity.

CHAPTER THREE

DETERMINATION OF MEPHENOXALONE IN BIOLOGICAL FLUIDS

3.1 INTRODUCTION

The quantitative measurement of drugs in biological fluids can rarely be applied directly to the sample matrices and purification procedures prior to chromatographic analysis are generally required (48). Sample preparation techniques and strategies for selecting an appropriate procedure for selectively isolating the compound of interest from biological fluids have been extensively reviewed (48,53-57). The most commonly employed extraction techniques are solvent extraction (liquid-solid or liquid-liquid) and separation with a solid additive or column packing material. The method employed will depend not only on the physico-chemical properties of the drug but also on the type of biological fluid being analyzed and the sensitivity requirements of the analysis. Biological factors such as protein-binding properties of the drug are also important since the drug may have to be freed from protein prior to extraction.

Two methods for isolating mephenoxalone from biological fluids, both liquid extraction procedures, have been reported (6,28). Eckhardt *et al.* (28) isolated the parent compound and several biotransformation products from human urine using chloroform as the organic solvent. The parent drug was extracted directly from urine while the unconjugated metabolites were extracted under acidic as well as basic conditions. Conjugated derivatives were subjected to acid/enzyme hydrolysis prior to extraction under acidic/basic conditions. The compounds were identified by TLC but no quantitative analyses were performed.

Morrison (6) isolated mephenoxalone from biological samples of laboratory animals using chloroform as the organic solvent. The addition of an equal volume of sodium hydroxide (1N) to plasma or urine (1-3ml) was followed by extraction with 10ml ethylene dichloride. Samples were centrifuged and the upper phase was removed by aspiration and discarded. The concentration of mephenoxalone in

the organic phase was then determined by spectrophotofluorimetry. The extraction efficiency for spiked samples was 85 to 100 percent for plasma and 90 to 100 percent for urine. The metabolites were not extracted and therefore not measured under these conditions.

In this study, a liquid-liquid extraction procedure was employed for the analysis of mephenoxalone in human serum and urine. The biological samples were assayed for unchanged drug by HPLC using UV detection at 200nm. HPLC involves a separation process and therefore lends itself to simplified techniques for sample preparation prior to analysis.

A protein-precipation step will often suffice provided that adequate detection sensitivity can be obtained. However when UV detection of 200nm is used it is essential that more rigorous sample preparation procedures be employed in order to improve the condition of the sample. The exclusion from the sample of endogenous components of the biological matrix not only provides the required selectivity but also affords protection of equipment from damage and deterioration. The use of a guard column before the main column is advocated for all biological fluid analyses to prolong the life of the analytical column (53).

A systematic approach to the development of a method for isolating mephenoxalone from human serum and urine is presented.

3.2 MATERIALS AND APPARATUS

3.2.1. Materials

As in Section 2.3.1

3.2.2 Source of Biological Samples

Drug-free human serum was obtained from the local blood transfusion centre and stored frozen at -20°C until required. The serum was thawed at room temperature before use.

Drug-free urine was freshly collected from a human volunteer on the day of the analysis.

3.2.3 HPLC System

The HPLC system used in this study has been previously described (Section 2.3.2). A 10μm C18 guard column (HPLC Technology Ltd., Chesire, England) was used before the main column.

3.2.4 Additional Equipment

As in Section 2.3.3.

3.3 DETERMINATION OF pKa OF MEPHENOXALONE

The dissociation constant of a drug is an important parameter in pharmacokinetic investigations. Once the pKa is known, the degree of dissociation of a compound at physiological pH can be easily determined (58) and, since only unionized molecules generally pass through biological membranes, predictions concerning the access of a drug to tissue sites can be made.

The advantage of knowing the pKa value prior to the development of a method for extracting a drug from biological fluids is obvious. In liquid-liquid extraction, partitioning of a compound into the organic phase is maximum when the drug is in its unionized form. The pH of the aqueous phase can therefore be manipulated to provide maximum drug recovery.

The accurate determination of the aqueous dissociation constant of an organic compound is routinely performed using potentiometric or UV spectrophotometric methods (59,60). These methods are not, however, applicable to the determination of pKa values of drugs, such as mephenoxalone, which are insufficiently soluble in aqueous media. Alternative methods can be used in such cases (61-68). The recent method of Hasegawa *et al.* (68) was used for this study.

3.3.1 Experimental

3.3.1.1 Sample Preparation

Aqueous buffer solutions (0.1 moles/litre, pH 3 and ph 5) containing mephenoxalone (0.06mg/ml) were prepared by dissolving 6mg quantities of mephenoxalone in phosphate buffer (see below) and making up to volume with buffer in 100ml volumetric flasks. The pH values were accurately adjusted using sodium hydroxide pellets with constant stirring on a magnetic stirrer, to produce two solutions of pH 3.00 $\pm$ 0.01 and pH 5.00 $\pm$ 0.01 respectively.

Phosphate buffer (0.1 moles/litre) was prepared by the addition of 3.35ml phosphoric acid to a 500ml volumetric flask and making up to volume with HPLC-grade water.

An internal standard stock solution was prepared as in Section 3.4.1.1. Appropriate dilutions of this solution were made with acetonitrile to provide the required concentration.

3.3.1.2 Determination of pKa

The partitioning of mephenoxalone between an aqueous and a water-immiscible organic solvent was investigated at two different aqueous phase pH values (3.00, 5.00). The organic solvents used in this study were methylene chloride and diethyl ether. Each sample was assayed in triplicate as follows.

Five millilitres of aqueous buffer solution containing mephenoxalone (see Section 3.3.1) and 5.0ml organic solvent were added to a screw-capped 15ml Kimax tube (Kimble Products, New Jersey, USA) and gently agitated on a mechanical reciprocating shaker for 3 hours. The tube was then centrifuged at 3000rpm for 5 minutes. Aliquots of 1.0ml each of the organic and aqueous phases were transferred to clean test tubes, to which 200µl of internal standard solution (0.3mg/ml phenacetin in acetonitrile) had been added. Ten microlitres of the aqueous sample mixture was injected into the HPLC system under the

conditions described in Section 2.4.2. The organic sample mixture was evaporated to dryness under a gentle stream of nitrogen at 37°C and the residue was reconstituted in 1.2ml HPLC-grade water. A 10µl aliquot was injected onto the column under the same conditions as above.

3.3.2 Results and Discussion

Table 3.1 shows the results obtained in this study. The pKa of mephenoxalone was found to be approximately 4.6 using either methylene chloride or diethyl ether as the organic phase. This value may not be representative of the true pKa value since there are many potential sources of error in the method employed:

(1) Methylene chloride and diethyl ether are partially miscible with the aqueous phase and, since the determination of pKa depends on the accurate measurement of drug concentrations in both the aqueous and organic phase, if there is phase miscibility, a deviation from the true result will be obtained. The use of paraffinic hydrocarbons have been advocated to minimize such an error (68).

(2) Hasegawa *et al.* (68) have demonstrated that both the drug concentration and the buffer concentration may alter the pKa value; the lowest possible concentrations have been advocated as optimal. However, these variables were not investigated in this study.

(3) To interpret the absorption and disposition of a drug in terms of pKa, the determination should be performed at 37°C (68). This study was however conducted at room temperature.

The low pKa values obtained in this investigation are in agreement with values that can be expected for mephenoxalone since the drug molecule contains a very weakly basic carbamate moiety. However, the results were not conclusive and a more extensive investigation of the variables that may affect the pKa of mephenoxalone would be necessary for the determination of the true value.

TABLE 3.1 pKa Values of mephenoxalone as determined by the partition method

ORGANIC PHASE	AQUEOUS PHASE pH	Pappa (SD)	Pmb	pKab
METHYLENE CHLORIDE	3.00	8.19 (0.34)	356.60	4.63
	5.00	6.10 (0.31)		
DIETHYL ETHER	3.00	0.85 (0.08)	39.68	4.66
	5.00	0.72 (0.08)		

a determined at ambient temperature

b calculated by the solution of simultaneous equations as described by Hasegawa *et al* (68).

3.4 SAMPLE EXTRACTION PROCEDURE

Data relating to concentrations of mephenoxalone in biological fluids of humans after therapeutic doses of the drug have not been published in the literature. The sensitivity requirements of the assay were therefore not known. Since drugs and their metabolites are usually present in serum and urine in low concentrations (55), the extraction method was directed towards providing maximum sensitivity.

3.4.1 Experimental

3.4.1.1 Sample Preparation

Mephenoxalone stock solution was prepared as follows: Fifty milligrams of mephenoxalone were dissolved in 25.0ml acetonitrile and 10.0ml of this solution was transferred to a 20ml volumetric flask and

made up to volume with HPLC-grade water to produce a 1mg/ml solution. Appropriate dilutions of this solution were made with water to provide the required concentrations.

Internal standard stock solution was prepared by dissolving 25mg phenacetin in 25.0ml acetonitrile to produce a 1mg/ml solution. A 50µg/ml solution was prepared by diluting 2.5ml of stock solution to 50.0ml with HPLC-grade water. This solution was used for the determination of mephenoxalone concentrations between 1.0 and 15.0µg/ml. For the analysis of concentrations between 0.1 and 1.0µg/ml, an internal standard solution containing 5µg/ml phenacetin was used. This was prepared by making a 1 in 10 dilution of the 50µg/ml solution with water.

Spiked serum and urine samples were prepared by the addition of appropriate amounts of a standard solution of mephenoxalone to drug-free biological fluid. Serum samples were spiked with mephenoxalone in the concentration range 0.1 to 15µg/ml. Urine samples were spiked with mephenoxalone in the concentration range 1 to 10µg/ml.

3.4.1.2 Chromatographic Conditions

The HPLC conditions employed throughout this study are described in Section 2.4.2.

3.4.1.3 Development of an Extraction Procedure

A preliminary investigation was conducted to examine the partitioning of mephenoxalone between 1.0ml water and 5.0ml aliquots of several common laboratory solvents. Thorough mixing of the biphasic solvent system, to which 5.0µg mephenoxalone had been added, was followed by centrifugation for 10 minutes at 3000rpm. The organic fraction was transferred to a clean collection tube and evaporated to dryness under a gentle stream of nitrogen at 37°C. The residue was reconstituted in a minimum volume of 200µl mobile phase and 10µl aliquots were injected into the liquid chromatograph. Ten microlitre aliquots of mephenoxalone standard solution (0.25µg/ml) were injected onto the column to provide a means of assessing the maximum recovery possible.

A selected range of organic solvents into which mephenoxalone exhibited favourable partitioning were then tested for their applicability to serum and urine extractions. These solvents included methylene chloride, chloroform, 1,2-dichloroethane, ethyl acetate and diethyl ether. Spiked serum samples (1.0ml) containing 5µg/ml mephenoxalone were extracted, as previously described, with 5.0ml aliquots of the organic solvents. The quality of each extract, in terms of extraction efficiency and cleanliness, was examined. A suitable organic solvent was thus selected for the determination of mephenoxalone in serum, after which an investigation of the partitioning of phenacetin, the internal standard, into the chosen solvent was conducted. The conditions for the extraction procedure were then optimized by examining the effects of changes in the volume ratio (serum:solvent), different mixing times, pH of the extracted sample, and reconstitution techniques.

The extraction procedure developed for the analysis in serum had to be modified for the determination of mephenoxalone in urine. The use of mixed solvents for extracting the drug was examined.

The extraction procedure developed for the analysis in serum had to be modified for the determination of mephenoxalone in urine. The use of mixed solvents for extracting the drug was examined.

3.4.2 Results and Discussion

3.4.2.1 Extraction of Aqueous Samples

A range of solvents for the preliminary partitioning investigation was selected on the basis of previous solvents that had been applied to the determination of mephenoxalone in biological fluids as well as other common laboratory solvents. However, only a few of the solvents tested had favourable mephenoxalone partitioning properties in the aqueous study. These comprised methylene chloride, 1,2-dichloroethane, chloroform, ethyl acetate and amyl alcohol. More that 70 percent of the amount of mephenoxalone added to the aqueous fraction was recovered in the organic fraction using these solvents.

The need to maximize the sensitivity of the method by concentrating the extracted organic fraction to a small end-volume meant that the non-volatile solvent, amyl alcohol, had to be excluded as a possible solvent. Chromatograms obtained from ethyl acetate extractions showed the presence of numerous contaminants, the source of which was ascertained to be the solvent itself; the evaporation to dryness of pure solvent followed by reconstitution and injection onto the column produced the chromatogram shown in Fig.3.1. The contaminant peaks do not, however, co-elute with mephenoxalone and this solvent was therefore not precluded as a possible extractant at this stage of the method development. The manipulative advantage of using ethyl acetate, which in contrast to the other solvents forms the upper phase of the aqueous-organic solvent system, is obvious.

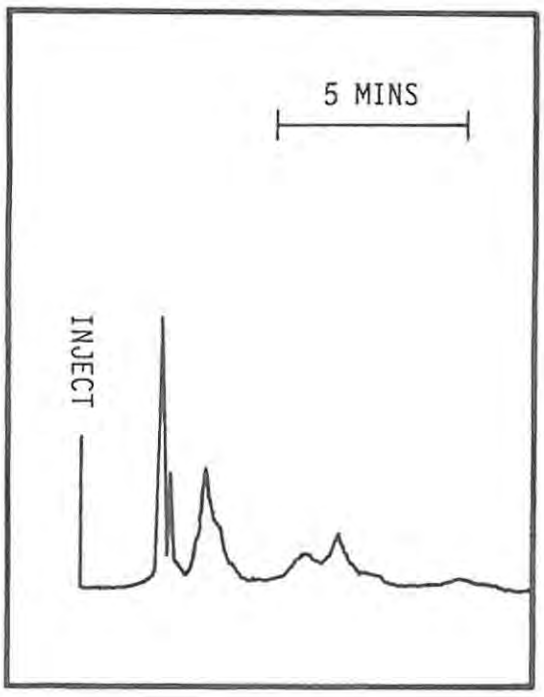


FIGURE 3.1 Chromatogram showing contaminaints present in ethyl acetate (chromatographic conditions as in Section 2.4.2; injection volume 10µl)

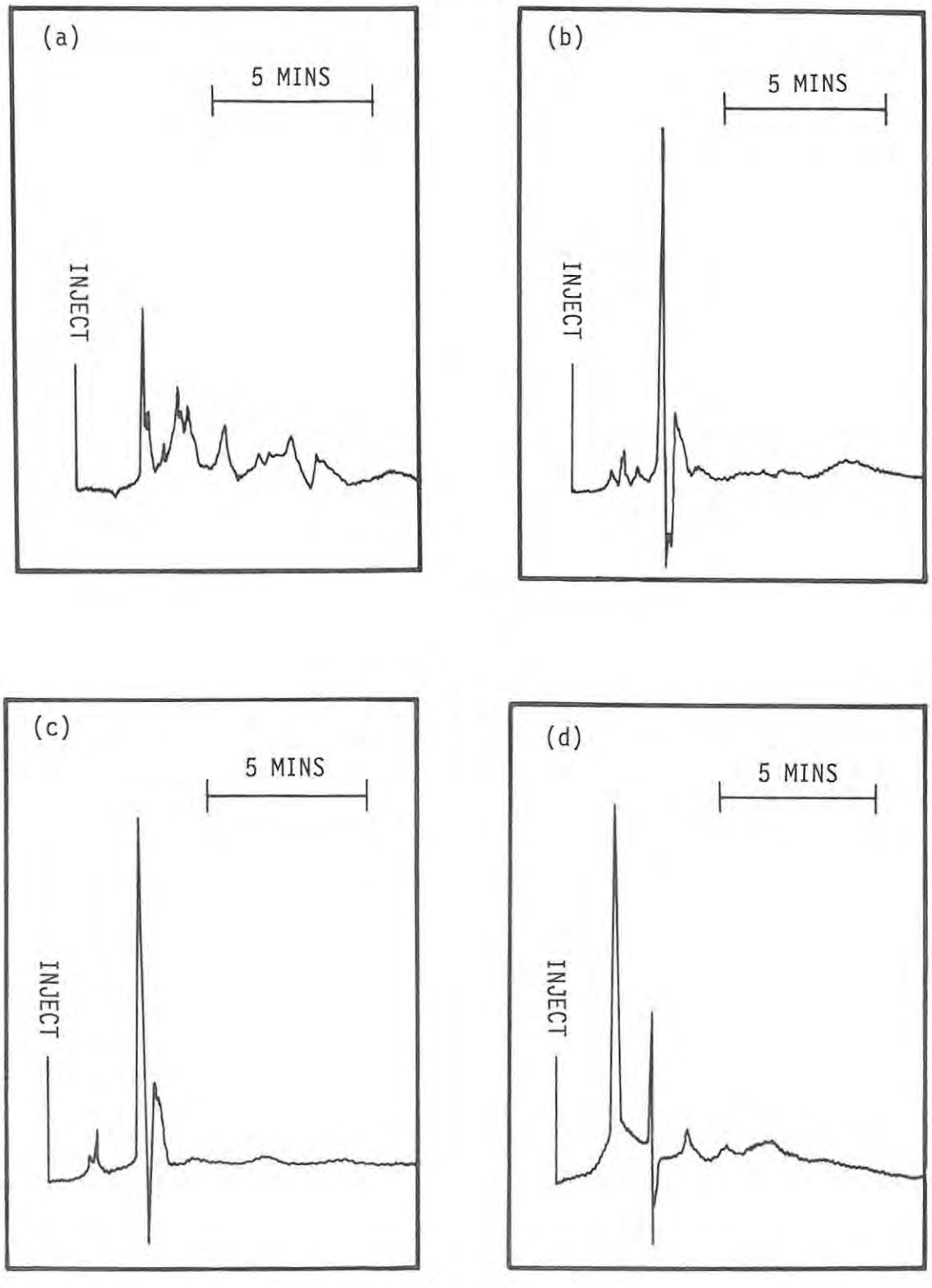
3.4.2.2. Extraction of Serum Samples

Preliminary extractions of drug-free serum samples (1.0ml) with ethyl acetate, chloroform, methlene chloride and 1,2-dichloroethane produced the chromatograms depicted in Fig.3.2. The extraction efficiency for mephenoxalone was more than 70 percent using these solvents but diethyl ether and ethyl acetate extracted numerous endogenous components from serum and were therefore excluded from further study. 1,2-Dichloroethane extracted more serum contaminants than did chloroform or methylene chloride.

Difficulties were experienced in penetrating the 'plug' that formed at the interface of the serum-chloroform solvent system. Plug formation was however reduced when serum proteins were precipitated prior to solvent addition. Protein precipitation was performed by the addition of 1.0ml acetonitrile to the 1.0ml serum sample, followed by centrifugation and transfer of the supernatant to a clean test tube. An extra manipulative step had, therefore, to be introduced. Another disadvantage of using chloroform was that it randomly formed emulsions when mixed with serum. The same effect was observed with 1,2-dichloroethane. Numerous methods for minimizing gel formation are cited in the literature (56,57) but have the disadvantage of additional sample manipulation steps.

Of all the solvents investigated, methylene chloride was the most suitable extractant in terms of cleanliness of the extract, lack of gel formation and relative ease of sample manipulation, and it was therefore employed for the routine determination of mephenoxalone in serum. The internal standard partitioned highly into methylene chloride, with approximately 85 percent of the added amount being recovered in the solvent. The optimum volume ratio (serum:organic solvent) was ascertained by increasing the organic volume until no further increase in drug recovery was obtained. A compromise between extraction efficiency and sample evaporating time was however necessary. The use of an 8.0ml aliquot of methylene chloride provided an extraction efficiency of 96.8 $\pm$ 3.8 percent whereas 5.0ml organic extractions extracted 92.2 $\pm$ 3.4 percent of the amount of mephenoxalone originally present in the sample. The time taken to

FIGURE 3.2 Chromatograms of drug-free serum extracted with (a) ethyl acetate, (b) chloroform, (c) methylene chloride and (d) 1,2-dichloroethane (chromatographic conditions as in Section 2.4.2; 10µl injection volume).



evaporate 8.0ml was however almost double that of the 5.0ml samples; the small increase in extraction efficiency that it provided did not warrant the use of an 8.0ml aliquot as the objective was to develop a rapid method whereby large numbers of samples could be routinely analyzed.

No significant increase in drug recovery was observed when spiked serum samples were vortex-mixed with methylene chloride for longer than 15 seconds. This value was therefore adopted as the standard vortex-mixing time for the analysis.

An investigation of the effect that sample pH changes had on extraction efficiency showed that the partitioning of mephenoxalone into methylene chloride was unaltered by either acidification or basification of the serum samples. In preliminary serum extraction studies, a decrease in drug recovery was observed when sodium hydroxide (1mole/litre) was added to the samples. This was found to be the result of drug entrapment in the emulsions that formed as a result of basificiation. When a protein precipitation step was included prior to solvent extraction, emulsification was no longer observed. The fact that sample pH does not affect the extraction efficiency may be of value in analyses which require co-extraction of other compounds of interest present in biological samples.

It was found that methylene chloride extracts which have been evaporated to dryness could be reconstituted in either water, mobile phase, acetonitrile or methanol without affecting drug recovery. It was initially thought that the insolubility of mephenoxalone in water might preclude this solvent as a possible candidate for reconstituting the dried extracts. However, at the concentrations employed in the study the compound was within the limits of its solubility in water and it was therefore employed for all further reconstitutions.

3.4.2.3 Extraction of Urine Samples

Extraction of urine with methylene chloride was found to yield a high mephenoxalone extraction efficiency but the chromatograms contained numerous contaminant peaks which interfered with the analysis

(Fig.3.3(b)). A typical chromatogram of a blank urine extract is shown in Fig.3.3(a). In addition the reconstituted extract was yellow in colour, possibly due to extraction by methylene chloride of various urine pigments. The use of a more non-polar extraction solvent, comprising a mixture of cyclohexane and methylene chloride (3:2 vol/vol), was found to dramatically improve the condition of the extract in terms of background interference. A typical chromatogram of a blank extract is shown in Fig.3.4. Using a 3:2 (cyclohexane:methylene chloride) volume ratio, a useful lowering of density was achieved and the organic mixture comprised the upper phase of the biphasic system. The manipulative advantage of such a system is obvious.

The internal standard was well extracted using the solvent and the extraction efficiency for mephenoxalone was approximately 80 percent, which was adequate for the determination of mephenoxalone in urine after oral administration of the compound.

3.4.2.4 Choice of Extraction Method for Serum and Urine

Liquid-liquid extraction is generally easy to perform, rapid and relatively economical. It is the ideal method if a single extraction provides a good recovery in terms of the amount and condition of the desired compound (56). The suitability and requirements of solvents employed in this technique have been reported in the literature (54,56). The final solvent choice depends not only on the recovery it affords but also on its purity, toxicity and cost. It is well-known that the least polar solvent that affords a reasonable drug recovery should be employed because it extracts minimal interfering sample components (48).

Liquid-liquid extraction processes are not without difficulties. Emulsion formation and adsorption of analyte molecules onto glassware comprise the most commonly encountered problems, both of which can be relatively easily minimized or avoided altogether (54,56).

FIGURE 3.3 Chromatograms of (a) an extract of drug-free urine and (b) an extract of urine containing 5.0µg/ml mephenoxalone[1] using 5.0 ml methylene chloride as the extraction solvent (chromatographic conditions as in Section 2.4.2; 10µl injection volume).

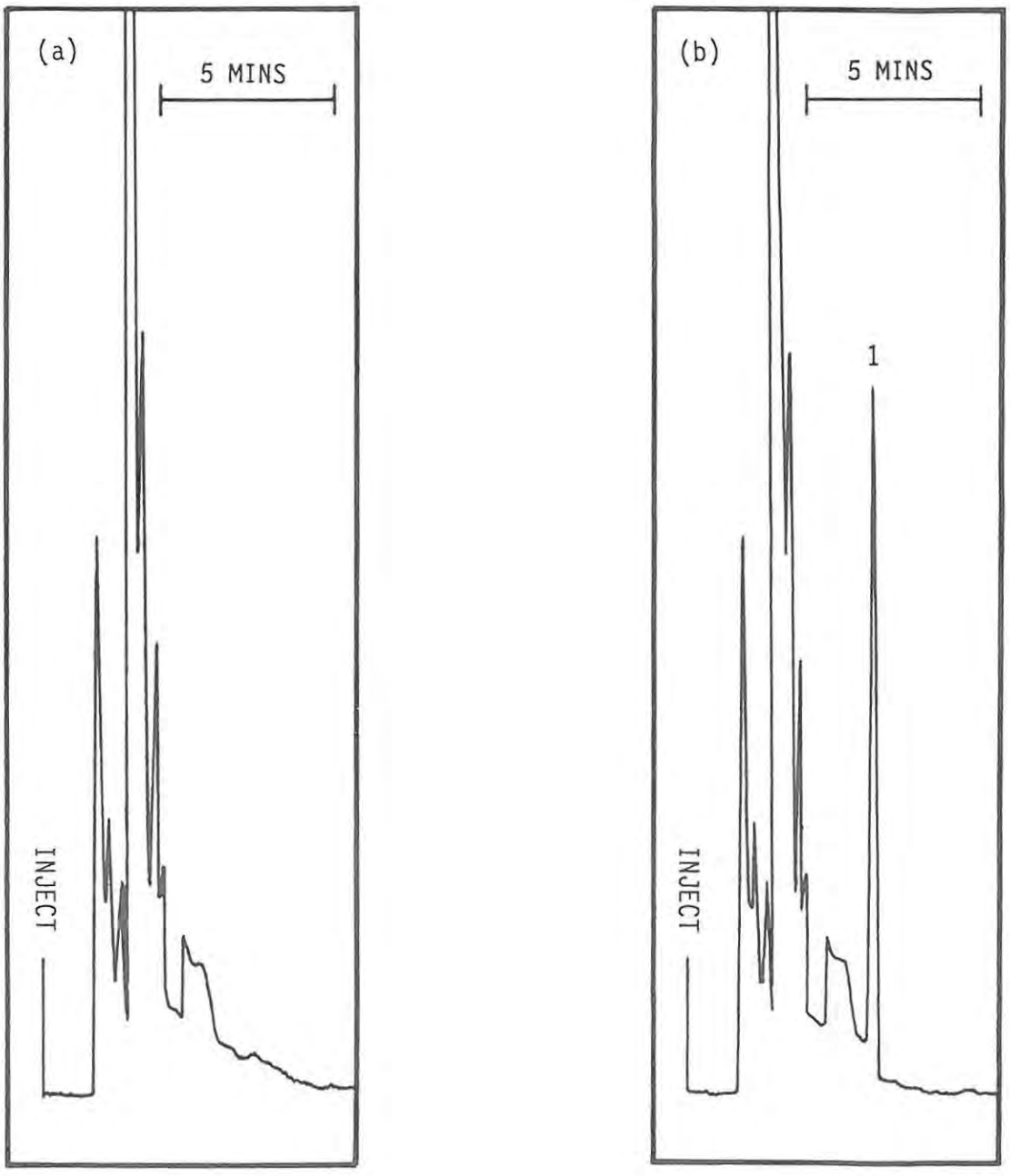
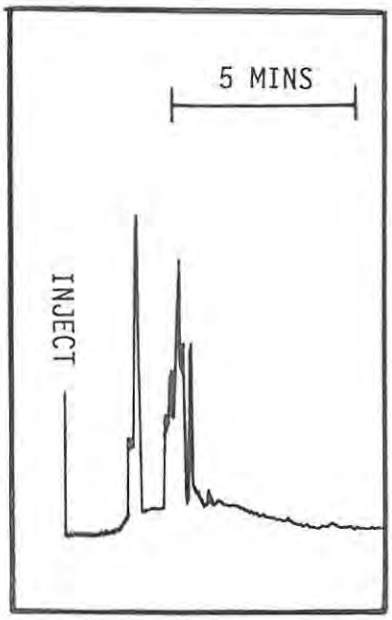


FIGURE 3.4 Chromatogram of an extract of drug-free urine using 5.0ml cyclohexane-methylene chloride (3:2) as the extraction solvent (chromatographic conditions as in Section 2.4.2; 10µl injection volume).



The liquid-liquid extraction methods reported in the literature employed chloroform and ethylene dichloride as the organic solvents (6,28). In this study, neither of these solvents were found to be suitable for mephenoxalone analysis in serum although chloroform could be used if steps were taken to prevent random emulsification of samples; the use of acetonitrile to precipitate the serum proteins was demonstrated to be effective. The use of 1,2-dichloroethane did not provide sufficiently clear extracts for HPLC analysis at 200nm. Methlene chloride was the solvent of choice for serum analysis for the following reasons:

(1) No emulsion formation was observed;
(2) The aqueous and organic layers were easily separated;
(3) A high extraction efficiency was obtained and the extract was sufficiently clean;
(4) The extraction was performed in a simple, rapid one-step process;

(5) The procedure was sufficiently sensitive for the routine determination of mephenoxalone in human serum and urine after oral dosing.

The above method was not however applicable to urine analyses because methylene chloride extracted numerous endogenous sample components. The use of a mixture of cyclohexane and methylene chloride (3:2) provided a sufficiently non-polar solvent to prevent the extraction of contaminants without significantly affecting the extraction efficiency, and this was therefore the solvent of choice for the routine estimation of large numbers of urine samples.

3.5 ANALYSIS OF MEPHENOXALONE IN SERUM

3.5.1 Experimental

3.5.1.1 Sample Preparation

Mephenoxalone stock solution (1mg/ml) was prepared as described in Section 3.4.1.1.

Internal standard solutions (5µg/ml and 50µg/ml) were prepared as described in Section 3.4.1.1.

A serum stock solution containing mephenoxalone was prepared as follows:
One millilitre of mephenoxalone stock solution (1mg/ml) was added to drug-free serum in a 50.0ml volumetric flask. After thorough mixing for 1 hour on a reciprocating mechanical shaker, appropriate dilutions of this solution were then made (Section 3.5.1.4), each diluted solution being mixed as before.

3.5.1.2 Chromatographic Conditions

As in Section 2.4.2.

3.5.1.3 Extraction Procedure

One millilitre of serum was mixed with 200µl internal standard solution (5µg/ml or 50µg/ml, Section 3.4.1.1.) in a test tube. Addition of 5.0ml methylene chloride was followed by vortex mixing for 15 seconds and centrifugation for 10 minutes at 3000rpm. The upper layer was removed by aspiration and discarded. A pasteur pipette was used to transfer the methylene chloride fraction to a clean test tube for evaporation to dryness under a gentle stream of nitrogen at 37°C. The residue was reconstituted in 200µl water and vortexed for 30 seconds to dissolve. Aliquots of 2 to 10µl were then injected onto the column.

3.5.1.4 Calibration Curves

Two calibration curves were constructed because of the wide range of concentrations of mephenoxalone found in serum after oral dosing; concentrations falling outside of the initial range of quantitation (1.0-15.0µg/ml) were found and it was thus necessary to re-validate the new calibration range (0.1-1.0µg/ml).

(a) Mephenoxalone serum concentration 1.0 to 15.0µg/ml

The serum stock solution (20µg/ml) was diluted to yield five different concentrations (15.0, 10.0, 5.0, 2.0 and 1.0µg/ml) each of which was assayed in triplicate.

(b) Mephenoxalone serum concentration 0.1 to 1.0µg/ml. The 1.0µg/ml mephenoxalone serum solution, prepared as above was diluted to yield four different concentrations (0.8, 0.5, 0.2, 0.1µg/ml), the 1.0µg/ml solution itself providing the fifth concentration. Each concentration was assayed in triplicate.

The calibration curves were constructed by plotting the ratios of peak height of mephenoxalone to that of the internal standard *versus* the respective mephenoxalone concentrations. A straight line fit of the data was made by least-squares linear regression analysis.

3.5.1.5 Precision

Within-run precision was assessed by extracting six spiked serum samples each at the highest and lowest mephenoxalone concentrations determined in the calibration curves. These concentrations were 15.0, 1.0 and 0.1µg/ml.

3.5.1.6 Extraction Efficiency

The analytical recovery of mephenoxalone from serum was assessed as follows:
Three different concentrations of spiked serum samples were extracted as described in Section 3.5.1.3 except that the internal standard was incorporated into the 200µl aliquot used in the final reconstitution step and was thus not carried through the extraction procedure. All samples were assayed in triplicate.

Standard solutions of mephenoxalone corresponding to those extracted above were taken to dryness under a gentle stream of nitrogen at 37°C. These samples were then reconstituted as for the serum samples, with the internal standard being included in the 200µl aliquot.

To determine the percentage recovery, the ratios obtained from the serum extracts were compared to those resulting from the equivalent concentrations of standard solutions.

3.5.2 Results and Discussion

3.5.2.1. Linearity and Calibration Curves

Linearity was established for both of the ranges of concentrations studied (0.1 to 1.0µg/ml and 1.0 to 15.0µg/ml). Details of the linearity data and the calibration curves for mephenoxalone in serum are shown in Table 3.2 and Fig.3.5, respectively.

Chromatograms of a blank serum extract and a serum extract containing mephenoxalone and internal standard are depicted in Figs.3.6 (a) and 3.6 (b), respectively. The retention time of mephenoxalone was 5.3 minutes and that of the internal standard (phenacetin), 6.6 minutes.

3.5.2.2 Precision

Results of the within-run precision study are depicted in Table 3.3. Relative standard deviations (RSD) at the upper and lower limits of the concentration range fall within the acceptable limits for drug determinations (56). The method, as indicated by the above results, was found to be reproducible.

3.5.2.3. Extraction Efficiency

Table 3.4 shows the analytical recoveries of mephenoxalone from serum. Results indicate that the recovery of mephenoxalone is constant and quantitative over the range of concentrations studied.

3.5.2.4 Sensitivity and Detection Limits

Under the conditions of this assay and based upon a signal-to-noise ratio of 3, the detection limit for mephenoxalone in serum was 0.04µg/ml. Although it was not necessary to increase the sensitivity of the determination, it could be increased by the use of a larger injection volume.

3.6 ANALYSIS OF MEPHENOXALONE IN URINE

3.6.1 Experimental

3.6.1.1 Sample Preparation

Mephenoxalone stock solution (1µg/ml) was prepared as in Section 3.4.1.1.

Internal standard solution (50µg/ml) was prepared as in Section 3.4.1.1.

TABLE 3.2 Linearity data for mephenoxalone in serum

CONCENTRATION RANGE (µg/ml)	SLOPE	Y-INTERCEPT	CORRELATION COEFFICIENT
0.1 to 1.0	1.7135	0.0571	0.9999
1.0 to 15.0	0.1612	0.06281	0.9993

FIGURE 3.5 Calibration curves for mephenoxalone in serum
(a) 0.1 to 1.0µg/ml (b) 1.0 to 15.0µg/ml

(a)

Sensitivity : 0.02 AUFS, recorder input 10 mV
Injection volume : 10µl

(b)

Sensitivity : 0.16 AUFS, recorder input 10mV
Injection volume : 2 - 10µl

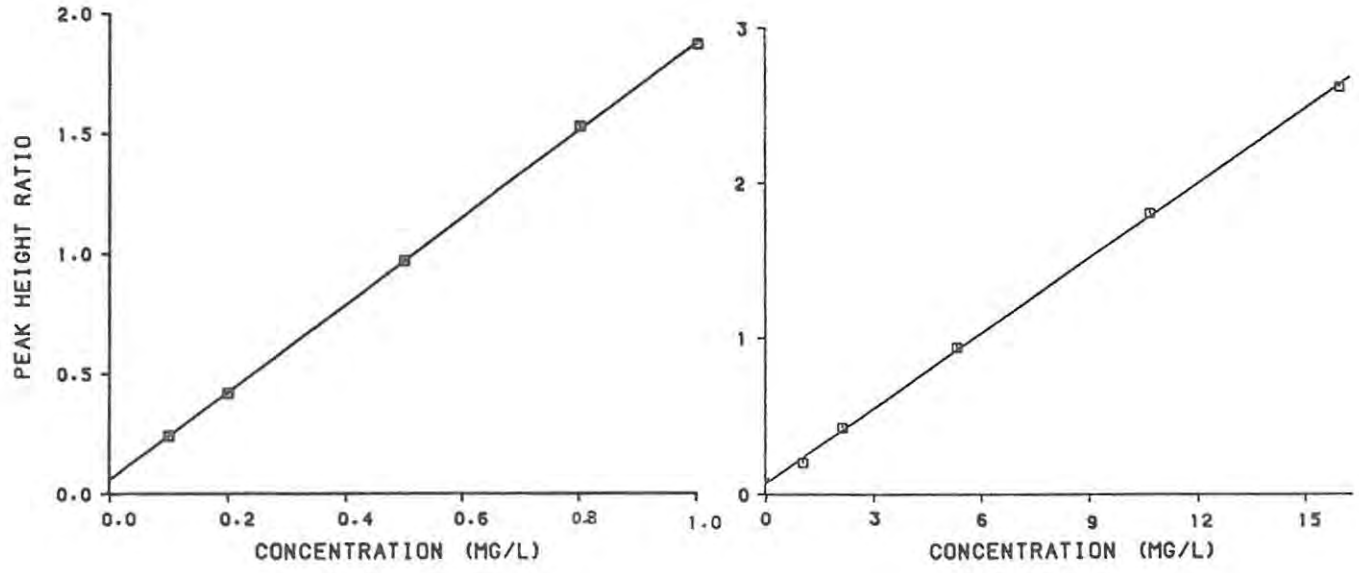
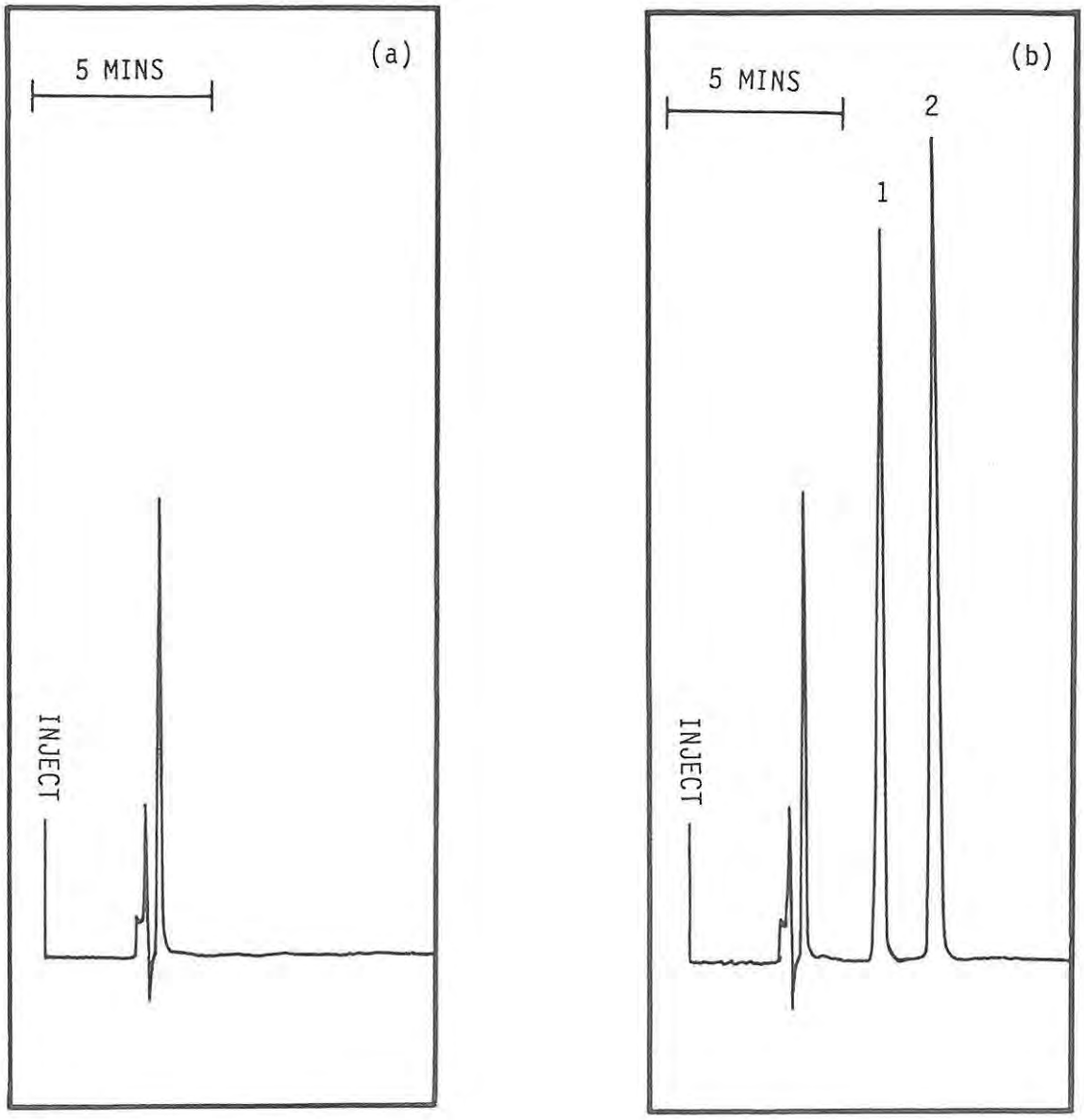


FIGURE 3.6 Chromatograms of (a) an extract of drug-free serum and (b) an extract of serum containing 5.0µg/ml mephenoxalone[1] and 5.0µg/ml phenacetin[2] (chromatographic conditions as in Section 2.4.2; 10µl injection volume).



Urine stock solution containing mephenoxalone (10µg/ml) was prepared as follows:

Five hundred microlitres of mephenoxalone stock solution (1µg/ml) was added to drug-free urine in a 50.0ml volumetric flask. Appropriate dilutions were then made.

3.6.1.2 Chromatographic Conditions

These were identical to those in Section 3.5.1.2. An attenuation of 0.16 AUFS was employed.

3.6.1.3 Extraction Procedure

One millilitre of urine was mixed with 200µl internal standard solution in a test-tube. Addition of 5.0ml cyclohexane-methylene chloride (3/2 vol/vol) was followed by vortex-mixing for 15 seconds and centrifugation for 10 minutes at 3000rpm. A pasteur pipette was used to transfer the organic extract to a clean test tube and the samples were then treated as for serum samples (Section 3.5.1.3). Aliquots of 10µl were injected onto the column.

3.6.1.4 Calibration Curve

The urine stock solution (10.0µg/ml) was diluted to yield four different concentrations, (8.0, 5.0, 2.0 and 1.0µg/ml), with the stock solution itself providing the fifth concentration. Each concentration was assayed in triplicate. A calibration curve was constructed as in Section 3.5.1.4.

3.6.1.5 Precision

Within-run precision was assessed by extracting six spiked urine samples each at the upper and lower limits of the concentration range studied. These concentrations comprised 1.0 and 10.0µg/ml.

TABLE 3.3 Within-run precision study on serum assay

CONCENTRATION RANGE (µg/ml)	SPIKED CONCENTRATION (µg/ml)	CONCENTRATION MEASURED (µg/ml) SAMPLE NUMBER 1	2	3	4	5	6	MEAN (SD)	RSD
0.1 to 1.0	0.1	0.11	0.11	0.10	0.11	0.11	0.11	0.11(0.003)	3.44%
	1.0	1.07	1.04	1.06	1.05	1.08	1.05	1.06(0.01)	1.27%
1.0 to 15.0	1.0	0.80	0.81	0.92	0.82	0.85	0.86	0.84(0.04)	4.78%
	15.0	16.33	15.63	15.60	15.67	15.81	15.76	15.80(0.25)	1.57%

TABLE 3.4 Analytical recoveries of mephenoxalone in serum

SERUM CONCENTRATION (µg/ml)	PERCENTAGE RECOVERY SAMPLE NUMBER 1	2	3	MEAN (SD)	RSD
0.1	92.9	90.5	88.0	90.5(2.0)	2.2%
1.0	90.4	90.1	87.3	89.3(1.4)	1.6%
15.0	88.2	87.1	89.5	88.3(1.0)	1.1%

3.6.1.6 Extraction Efficiency

The analytical recovery of mephenoxalone in urine was assessed in a manner similar to that described in Section 3.5.1.6.

3.6.2 Results and Discussion

3.6.2.1 Linearity and Calibration Curve

Linearity was established for the range of concentrations studied (1.0 to 10.0µg/ml). Details of the linearity data and the calibration curve for mephenoxalone in urine are shown in Table 3.5 and Fig.3.7, respectively.

Chromatograms of a blank urine extract and a urine extract containing mephenoxalone and internal standard are depicted in Figs.3.8 (a) and 3.8 (b), respectively.

Initial studies using methylene chloride for extracting urine samples showed that linearity existed over the range of concentrations studied (1.0 to 10.0µg/ml). The calibration curve had a slope of 0.1687, a y-intercept at 0.0026 and a correlation coefficient of 0.9953. Chromatograms of a blank urine extract and a urine extract containing mephoxalone are depicted in Figs.3.3 (a) and 3.3 (b), respectively (Section 3.4.2.3). By comparing the correlation coefficient obtained using methylene chloride alone, the calibration curve using cyclohexane-methylene chloride (3 :2) had a better correlation coefficient (0.9987) because the extracts obtained using the latter extracting solvent contained no contaminants that co-eluted with mephenoxalone. The mixed solvent was therefore adopted throughout this study.

3.6.2.2 Precision

Results of the within-run precision study are shown in Table 3.6. The relative standard deviations (RSD) at the concentrations studied fall within the acceptable limits for drug determinations (56).

TABLE 3.5 Linearity data for mephenoxalone in urine

CONCENTRATION RANGE (µg/ml)	SLOPE	Y-INTERCEPT	CORRELATION COEFFICIENT
1.0 to 10.0	0.1536	-0.0018	0.9987

FIGURE 3.7 Calibration curve for mephenoxalone in urine (1.0 to 10.0µg/ml)

Sensitivity : 0.16 AUFS, recorder input 10mV
Injection volume : 10µl

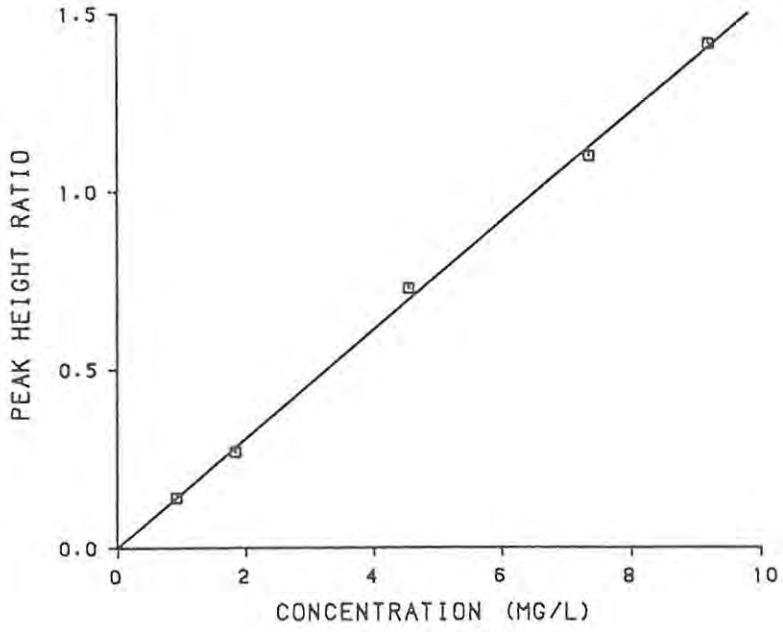
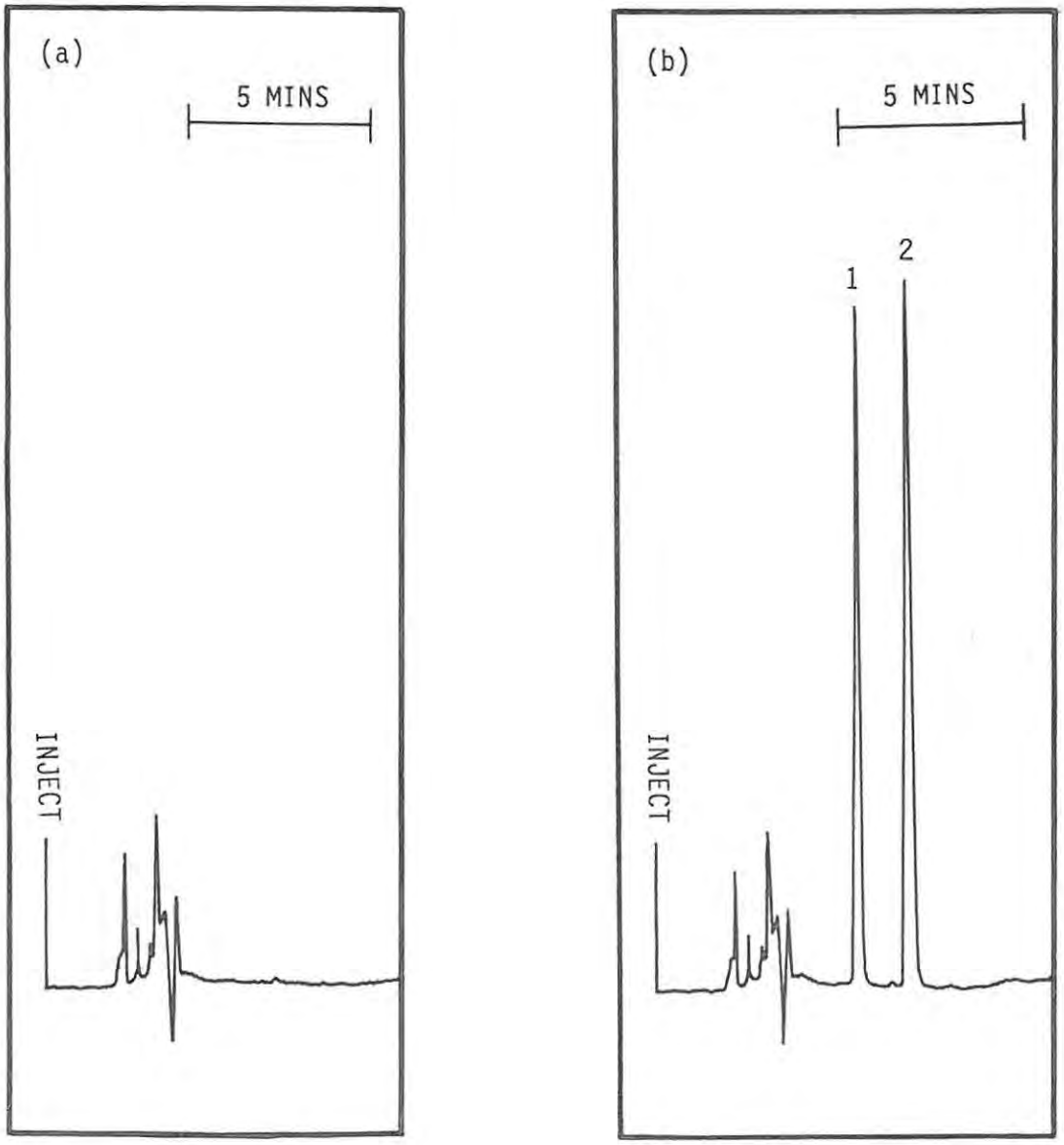


FIGURE 3.8 Chromatograms of (a) an extract of drug-free urine and (b) an extract of urine containing 5.0µg/ml mephenoxalone[1] and 5.0µg/ml phenacetin[2] using 5.0 ml cyclohexane-methylene chloride (3:2) as the extraction solvent (chromatographic conditions as in Section 2.4.2; 10µl injection volume).



3.6.2.3 Extraction Efficiency

Table 3.7. shows the analytical recoveries of mephenoxalone from urine. The mean recovery was 80.2 percent which is well within the accepted limits for drug determinations (56). The mean recovery for urine extracted with methylene chloride was 89.8 percent.

3.6.2.4 Sensitivity and Detection Limits

The minimum detectable limit for mephenoxalone in urine, using the conditions described previously, was 0.2µg/ml. This was lower than the sensitivity required for the determination of mephenoxalone in human urine after oral dosing.

TABLE 3.6 Within-run precision study on urine assay

SPIKED CONCENTRATION (µg/ml)	CONCENTRATION MEASURED (µg/ml)						MEAN (SD)	RSD
	SAMPLE NUMER							
	1	2	3	4	5	6		
1.0	0.93	0.92	0.92	0.91	0.92	0.94	0.92(0.01)	1.02%
10.0	9.28	9.19	9.22	9.26	9.20	9.14	9.22(0.05)	0.50%

TABLE 3.7 Analytical recoveries of mephenoxalone in urine

URINE CONCENTRATION (µg/ml)	PERCENTAGE RECOVERY			MEAN (SD)	RSD
	SAMPLE NUMBER				
	1	2	3		
1.0	78.0	79.4	80.7	79.4(1.1)	1.4%
10.0	81.2	79.5	82.1	80.9(1.1)	1.3%

3.7 STABILITY OF SERUM AND URINE SAMPLES ON STORAGE

The stability of a compound in biological fluids is an important aspect of drug development. The majority of stability studies reported in the literature do not, however, adequately describe either the experimental procedure or the statistical treatment of the data. Some procedures entirely avoid statistical treatment and judge stability on the basis of a measured response difference, D, between stored and freshly prepared samples (69-71). Several studies have reported the use of a *t*-test to ascertain whether any significant difference exists between the stored and freshly prepared samples (72-75). These methods do not consider the precision or number of measurements and are unable to distinguish between a significant and a pharmacokinetically relevant degradation. A novel approach for dealing with the stability of drugs in biological fluids which account for these factors has been recently reported (76). The procedure is based on sound experimental design and allows conclusions to be drawn concerning drug stability with an acceptable degree of certainty.

The application of this method to determine the stability of mephenoxalone in human serum and urine was evaluated for various drug concentrations, storage temperatures and storage times. The samples tested comprised spiked serum and urine samples as well as samples collected from human voluteers following oral administration of mephenoxalone.

3.7.1 Experimental

3.7.1.1 Sample Preparation

Mephenoxalone stock solution (1mg/ml) was prepared as in Section 3.4.1.1. Appropriate dilutions of this solution were then made with water.

Internal standard stock solution (1mg/ml) was prepared as in Section 3.4.1.1. A 50µg/ml solution was prepared as previously described.

Mephenoxalone spiked serum samples (1 and 10µg/ml) were prepared as follows:
One millilitre of mephenoxalone stock solution was diluted to 100.0ml with blank serum to provide a concentration of 10µg/ml. One millilitre of a 1 in 10 dilution of mephenoxalone stock solution was added to a 100ml volumetric flask and made up to volume using blank serum. This solution had a concentration of 1µg/ml.

Mephenoxalone spiked urine samples (1 and 10µg/ml) were prepared in a similar manner to the mephenoxalone spiked serum samples.

Serum and urine samples were collected from human volunteers as described in Section 5.1.1.6 and Section 5.1.1.7, respectively.

3.7.1.2 Storage Conditions

Six millilitre aliquots of the spiked serum and urine samples were stored under the following conditions:

Storage temperature		Period of Storage
Ambient	:	24 h
4°C	:	24 h, 48 h, 1 week
-20°C	:	1, 4, 8 weeks

Six millilitre aliquots of urine collected from human volunteers after oral dosing were stored as above but an insufficient volume of serum meant that only 3.5ml aliquots were stored, the conditions being the same as above.

3.7.1.3 Chromatographic Conditions

As in Section 3.5.1.2

3.7.1.4 Extraction of Serum and Urine Samples

Serum and urine samples were analyzed as described in Section 3.5.1.3 and Section 3.6.1.3 respectively.

3.7.1.5 Statistical Interpretation of the Data

The data were treated according to the procedure described by Timm *et al.* (76). The measured response difference (D) and the true percentage difference in response (Δ) between stored and freshly prepared samples were calculated by applying the equations given in Table A3.1. The true percentage change of concentration may differ from D because of the imprecision of the analytical method. The true change of response on storage was enclosed by a lower limit (LL) and an upper limit (UL) of a 90% confidence interval, with a probability of 5% that it was lower than LL and 5% that it was higher than UL. A change of response was considered pharmacokinetically relevant when a degradation of Δ =-10% was found. The relationship between significant and relevant changes of response on storage is shown in Fig.3.9.

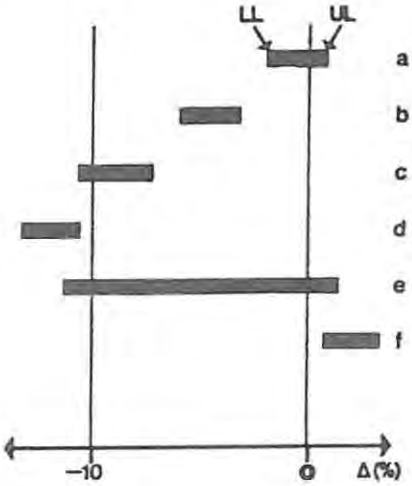


FIGURE 3.9 The relationship between significant and relevant change of response on storage. The bars above the axis characterize the ranges of the 90% confidence intervals for the true percentage response differences, Δ, between stored and freshly prepared samples. (LL = lower limit; UL = upper limit of the confidence interval); (a) change of response, not significant and not relevant; (b) decrease of response, significant but not relevant; (c) decrease of response, significant and possibly relevant; (d) decrease of response significant and relevant; (e) decrease of response, not significant but possibly relevant; (f) increase of response, significant.

Reprinted from Timm *et al.* (76)

3.7.1.6 Stability of Mephenoxalone in Serum and Urine on Storage

On the date the stored samples were to be analyzed, freshly prepared samples were made up from fresh stock solutions using the same mephenoxalone substance batch and biological fluid batches as were used for the stored samples. Five 1.0ml aliquots of the freshly spiked samples were analyzed together with five 1.0 ml samples of the stored serum and urine according to the procedure previously described. Calibrators were analyzed at the same time (as in Sections 3.5.1.4 and 3.6.1.4)

The measured responses (peak height ratios mephenoxalone:internal standard) were converted to concentrations using the calibration data. The statistical treatment was then applied to the concentrations of the stored and freshly prepared samples. The measured response difference (D) and the true percentage difference in concentration after storage ($_{\Delta}$) were calculated for each storage condition investigated.

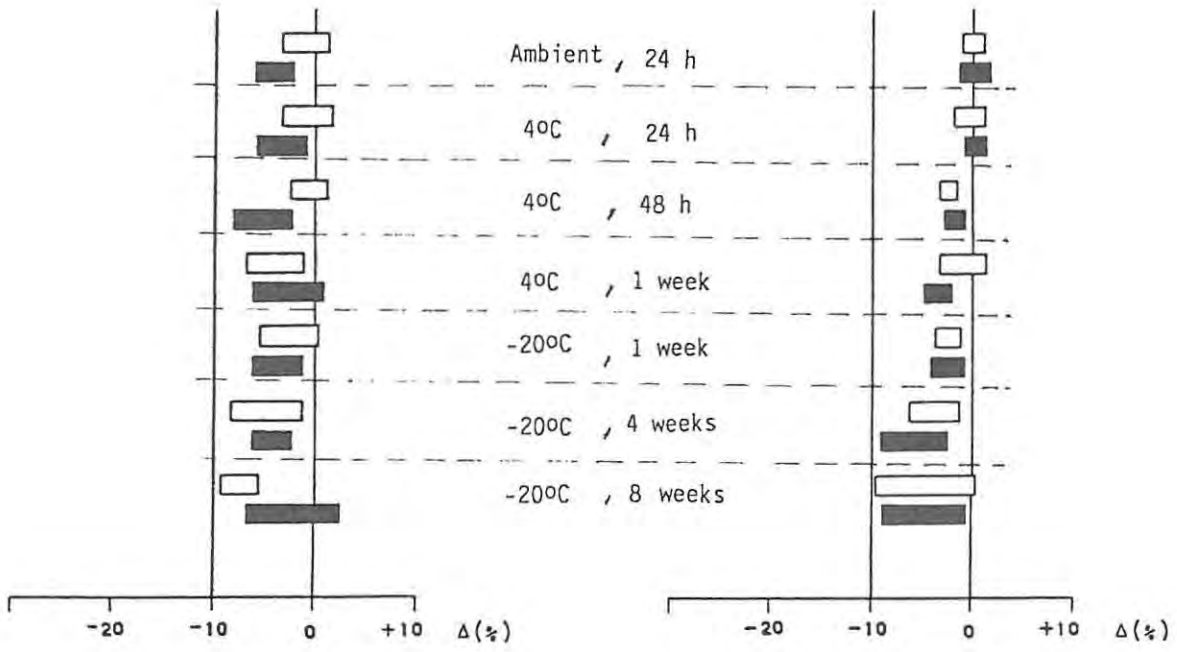
3.7.2 Results and Discussion

A detailed example demonstrating the statistical calculations for assessing the stability of mephenoxalone in serum and urine is presented in Table A3.2. The results from the studies of mephenoxalone stability in biological fluids are shown in Fig.3.10. When mephenoxalone was stored in human serum (spiked and clinical trial samples) under the conditions tested, no significant decrease in concentration was detected. However, mephenoxalone stored in both spiked and clinical trial urine samples showed a trend towards instability when stored for 4 weeks at -20°C. The instability appeared to be concentration-independent. According to the definition of Timm *et al.* (76), the decrease of response was significant and possibly relevant. A relevant degradation of 10 per cent or more could not be excluded with a statistical probability of 95%, and the drug was therefore not stable under these conditions.

FIGURE 3.10 Stability of mephenoxalone in human serum and urine
(■ concentration of 10µg/ml; □ concentration of 1µg/ml)

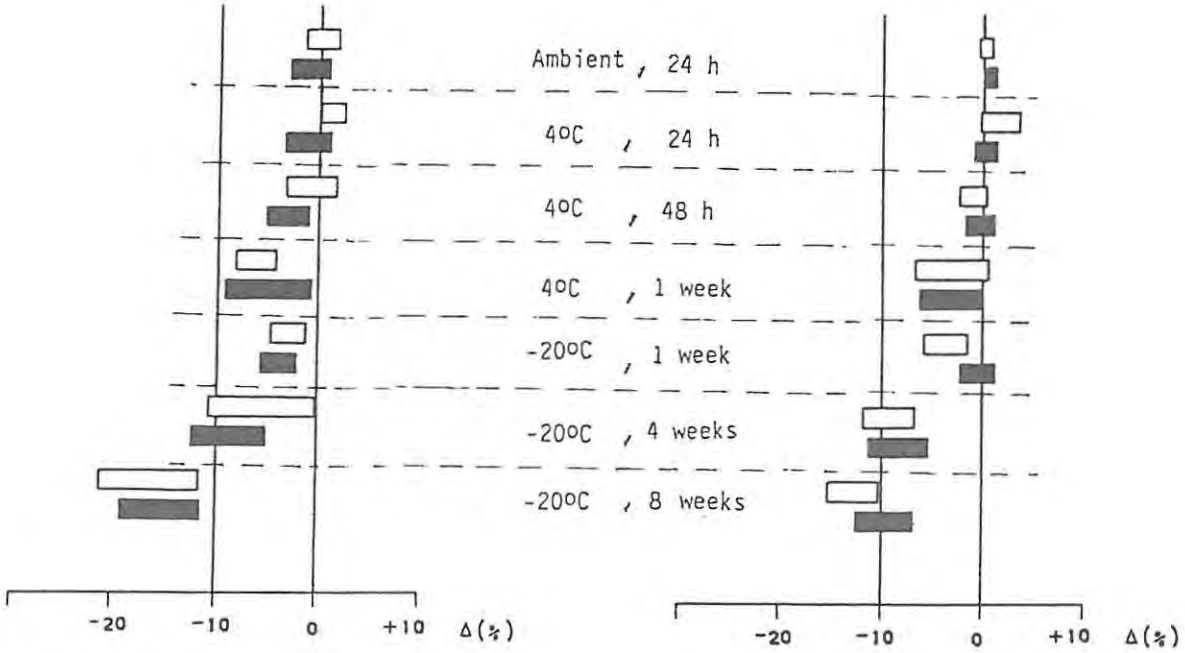
(a) Spiked serum samples

(b) Serum samples collected after oral administration of the drug



(c) Spiked urine samples

(d) Urine samples collected after oral administration of the drug



A significant and pharmacokinetically relevant decrease in concentration was observed when mephenoxalone was stored in spiked urine samples for 8 weeks at -20°C. Therefore, the time for storing urine samples at -20°C should be limited to 1 week. Serum samples on the other hand, may be stored for 8 weeks at -20°C without any significant or relevant degradation taking place.

Using the procedure described by Timm *et al.* (76), a comprehensive study of the stability of mephenoxalone in human serum and urine under various storage conditions was facilitated. The procedure accounted for the precision of the measurements and the number of replicate determinations performed. The presentation of the data obtained was simple and permitted a distinction to be made between pharmacokinetically relevant and statistically significant degradation.

3.8 PLASMA PROTEIN-BINDING OF MEPHENOXALONE

Drug binding to plasma proteins is an important biological factor of pharmacological and pharmacokinetic significance (77-79). Only the free drug, unbound by protein, is available for membrane transport. Bound drug is therefore not available for tissue distribution, receptor interaction or excretion. However, it is only when the extent of binding is high and when the drug-protein complex formed is not readily dissociable that binding becomes significant in the practical sense (78). The importance of determining the extent of protein-binding of a drug is thus obvious.

The binding properties of mephenoxalone have not been reported in the literature, probably due to the lack of a sensitive and precise analytical procedure for the measurement of the drug in biological fluids. The newly developed HPLC method for the assay of mephenoxalone was applied to the determination of mephenoxalone protein-binding. The extent of binding was determined by equilibrium dialysis.

Equilibrium dialysis involves the equilibration of unbound drug between a protein solution (plasma/serum) and a protein-free solution

(buffer) separated by a semi-permeable membrane. It is now known that volume shifts occur during dialysis from the buffer side to protein side of the membrane as a consequence of osmotic differences in the fluids (81, 82). The free drug fraction has conventionally been calculated as the ratio of the drug concentration in the buffer to that in the plasma, the volume shift being ignored (83). This results in an under-estimation of the true binding extent. Several methods have been proposed for correcting the volume shift that occurs during dialysis (80-82, 84). The most applicable to this study is the method of Sun *et al.* (80) because the volume shift is quantitated and incorporated into the calculation of the true extent of binding without any need to know the concentration of protein.

3.8.1 Experimental

3.8.1.1 Sample Preparation

Serum samples spiked with mephenoxalone (0.5 to 15.0µg/ml) were prepared as described in Section 3.5.1.1.

Clinical trial serum samples were collected from Subject KZ at 1.17, 3.0 and 6.0 hours after oral administration of two tablets, each containing 200mg mephenoxalone (see Section 5.1.1.6).

3.8.1.2 Equibilibrium Dialysis

Equilibrium dialysis was performed using two-chambered dialysis cells, each of 1.0 ml capacity (The Chemical Rubber Company, Ohio, USA). The cells were separated by a semi-permeable membrane (Spectra/Por 2, Spectrum Medical Industries, Inc., Los Angeles, Cal., USA) with a molecular weight cutoff of 12 000 - 14 000amu. Prior to use the membranes were boiled for 90 minutes in distilled water and stored for at least 24 hours at 4°C in phosphate buffer (0.1 moles/litre; pH 7.4).

Human serum (1.00ml) containing mephenoxalone was one half-cell while phosphate buffer solution (0.1 moles/litre, pH 7.4) of an equal volume was placed in the other. The dialysis cells were then left on a

reciprocating mechanical shaker at ambient temperature to equilibrate for 24 hours (prolonged incubation was verified not to change the plasma dialysate concentrations further). Gentle mixing was further facilitated by the inclusion into each half-cell of two glass beads. After equilibration the volumes of plasma and dialysate were accurately measured using a 2.0ml micro-volumetric syringe (Hamilton Co., Reno, Nev., USA). Aliquots of 750µl were transferred to clean test-tubes and prepared for HPLC analysis as described in Section 3.5. The dialysis procedure was performed in triplicate for each sample concentration.

3.8.1.3 Calculation of Protein-Binding

The degree of mephenoxalone binding to serum proteins (f_B) was calculated using Equation 3.1 (80).

$$f_B = 1 - \frac{C_F(1- V)}{C_T(1+ V)} \qquad \text{Equation 3.1}$$

where C_F is the unbound drug concentration, and
C_T is the total drug concentration after equilibration.
V is the fractional increase of plasma volume after equilibration.

3.8.2 Results and Discussion

The mean data for mephenoxalone binding to human serum proteins for spiked and clinical trial samples are presented in Table 3.8.

A comparison of the percentage bound when volume changes were included (Method A) and when the changes were excluded (Method B) from the binding calculations is shown. Using Method B, the true extent of binding was under-estimated, the difference being greater at the lower binding levels, thus demonstrating the importance of monitoring volume shifts.

The volume loss from plasma samples during dialysis ranged from 10 to 18 percent of the original volume (1.00ml). The recovery of samples from the dialysis cells was almost complete, with more than 96 percent of the volume added being recovered after dialysis.

A graphical presentation of mephenoxalone-binding to human serum proteins using Method A (Fig.3.11) showed that mephenoxalone exhibited a relatively concentration-independent change in the extent of binding for both the spiked and the trial samples. The sharp increase in binding from 50 percent at 0.5µg/ml up to 88 percent at 2.0µg/ml and subsequent decline to 50 percent bound at 10.0µg/ml for spiked serum samples has not yet been explained. Whether these results were true or produced by methodological artefacts is not known. Factors other than volume shifts have been shown to influence the *in-vitro* protein-binding of some drugs during dialysis (82, 84). These include the Donnan effect, non-specific binding of the drug to membranes, and pH differences between protein and buffer solutions. The effects of the above were not investigated.

Although the results obtained in this study were not conclusive in any way, an examination of the chemical structure and physico-chemical properties of mephenoxalone did provide some insight into the possible binding properties of the drug.

Mephenoxalone is a low molecular mass, hydrophobic, water-insoluble compound, and is therefore likely to be considerably bound to plasma proteins (77). The compound is not, however, expected to be tightly bound to these proteins because the forces involved in the binding are likely to be due only to lipophilic attractions of the aromatic ring (pi) electrons and the non-polar alkyl chains of the drug molecule (78). The compound has a very weakly basic carbamate moiety. For the above reasons it is expected that the binding of mephenoxalone to serum proteins, although reasonably high, is readily dissociable and therefore without clinical importance.

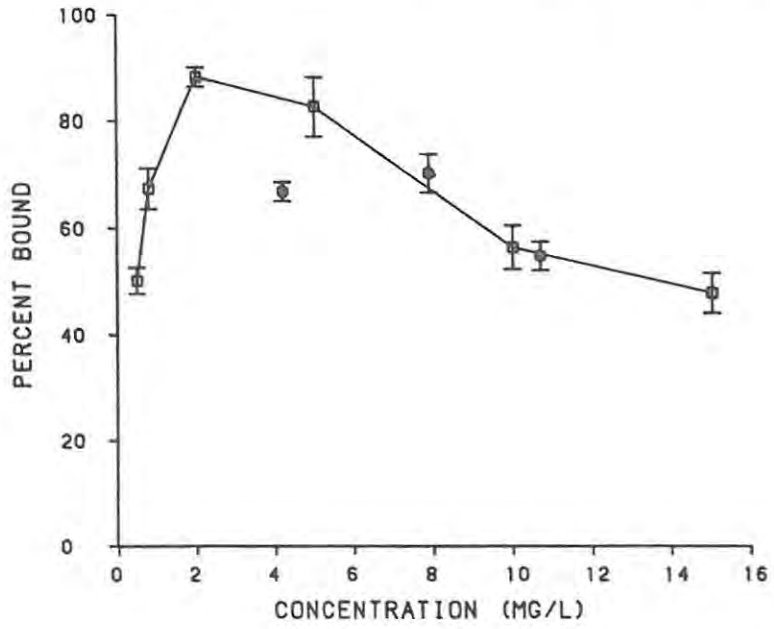
TABLE 3.8 Binding of mephenoxalone to serum proteins

	CONCENTRATION (µg/ml)	METHOD A[a] % BOUND (SD)	METHOD B[b] % BOUND (SD)
SPIKED	0.5	50.2 (2.5)	27.3 (4.4)
SAMPLES	0.8	67.4 (3.8)	51.0 (4.8)
	2.0	88.3 (1.0)	80.9 (1.1)
	5.0	82.6 (5.6)	75.1 (7.8)
	10.0	56.4 (4.1)	42.9 (3.6)
	15.0	47.8 (3.8)	34.7 (5.1)
CLINICAL	4.2	66.8 (1.1)	63.0 (4.9)
TRIAL	7.9	70.2 (3.6)	63.6 (4.4)
SAMPLES	10.7	54.8 (2.7)	38.8 (1.8)

a Volume change accounted for, calculated using Equation 3.1

b Volume change not accounted for, calculated according to method of Bender *et al.* (83)

FIGURE 3.11 Protein-binding of mephenoxalone in (▫) spiked serum samples and (●) serum samples collected after oral administration of a 400 mg dispersion of the drug.



4.2 EXPERIMENTAL

4.2.1 Conditions for Dissolution Testing

Dissolution apparatus	: as in Section 4.1.2, and set up according to the specifications in the USP XXI (Apparatus 2) (85)
Rotation rate of the paddles	: 100 rpm
Temperature of dissolution medium	: 37°C
Sample withdrawal position	: midway between the shaft of the paddle and the side of the containing vessel and half the distance from the surface of the medium to the bottom of the paddle blade.
Dissolution media	: 3 dissolution media were used for this study: (a) HPLC - grade water (b) Simulated gastric fluid (GF) (c) Simulated intestinal fluid (IF)

GF and IF were prepared according to the USP (86) with the exception that pepsin and pancreatin, respectively, were excluded. One hundred milligrams of methocarbamol, as the internal standard, was included in every litre of dissolution medium. The solutions were degassed and filtered through a 0.45µm membrane filter (Type HVLP, Millipore Corp., Bedford, Mass., USA) prior to use.

4.2.2 Chromatographic Conditions

The chromatographic conditions employed throughout this study are described in Section 2.4.2.

4.2.3 Tablet Assay

No official monographs on mephenoxalone dosage forms appear in the BP or the USP, thus no methods are specified for their analysis. However, the manufacturer of the new mephenoxalone-containing tablet

has proposed an assay method (2), and although the method was used for this study, it was adapted to include an internal standard as well as an HPLC analytical technique.

a) Standard Solution

Two hundred milligrams of mephenoxalone powder was accurately weighed into a 100ml volumetric flask to which was added 60ml methanol containing 0.1mg/ml methocarbamol as the internal standard. The drug was dissolved with the aid of ultrasonication and the resulting solution was made up to volume with the methanolic internal standard solution. Five millilitres of this solution were pipetted into a 100ml volumetric flask and made up to volume as before. Aliquots of 5µl were injected onto the HPLC column.

b) Sample Preparation

Twenty tablets were weighed, finely crushed, and a mass of powder approximately equivalent to the weight of one tablet was accurately weighed into a 100ml volumetric flask. The procedure described under "standard solution" (a) was then followed. The final suspension was sonicated once more and then filtered through a 0.45µm filter (Type HVLP, Millipore Corp., Bedford, Mass., USA). The first part of the filtrate was discarded and 5ul aliquots of the subsequent filtrate were injected onto the HPLC column. The tablet assay was carried out in triplicate using further portions of the powdered tablets.

4.2.4 *In Vitro* Dissolution Study

4.2.4.1 Intrinsic Dissolution of Mephenoxalone Powder

Initially, three different media were evaluated for their effect on the dissolution of 200mg pure mephenoxalone powder:

(i) 1000ml HPLC-grade water
(ii) 1000ml GF
(iii) 1000ml IF

Two hundred milligrams of powder was placed in the dissolution medium and the rotating paddle was immediately lowered into the medium. Using a microvolumetric syringe (Hamilton Co., Reno, Nev., USA) 100µl samples were withdrawn at 10 minute intervals and transferred to clean, limited volume WISP inserts. Five microlitre aliquots of the samples were then injected onto the HPLC column under the conditions described in Section 4.2.2. The peak height ratios (mephenoxalone/internal standard) were used in conjunction with an aqueous calibration curve to determine the amount of drug dissolved. Three dissolution runs were carried out for each medium.

4.2.4.2 Dissolution of Mephenoxalone-containing Tablets

At the time of this study, a limited supply of the new tablet dosage form was available. The dissolution study was therefore conducted only in simulated gastric fluid. Six tablets were assayed as follows. Each tablet was placed in 1000ml dissolution medium, immediately after which the rotating paddle was lowered into the medium. Samples of 100µl were withdrawn at 10 minute intervals as before, transferred to clean, limited volume WISP inserts and then injected (5µl aliquots) onto the HPLC column. The peak height ratios were used to determine the amount of drug dissolved, as before. The samples, which had been collected over a 2 hour period, were re-injected at the end of the HPLC run to ascertain whether or not any undissolved material that may have been withdrawn with the samples had dissolved upon standing in the WISP inserts.

4.3 RESULTS AND DISCUSSION

The mephenoxalone content of the tablet dosage form, as determined by HPLC analysis, was found to be 98.7 $\pm$ 1.0% of the stated amount. The HPLC method was rapid and accurate, and neither codeine phosphate nor paracetamol (both of which were also present in the dosage form) interfered with the determination of mephenoxalone or of methocarbamol; under the conditions of the analysis codeine was retained on the column, while paracetamol eluted prior to the compounds of interest.

Methocarbamol was chosen as the internal standard for reasons discussed in Section 2.5.6 and because of its favourable elution time. Incorporation of the internal standard into the dissolution medium had numerous associated advantages. In addition to compensating for any loss in the total volume of dissolution fluids due to evaporation during the dissolution run and from the withdrawn sample, it enabled the sample to be injected directly onto the column without additional manipulative steps. The dissolution characteristics of mephenoxalone powder were not affected by the presence of the internal standard as shown by the fact that identical profiles were obtained when the internal standard was added after sampling.

Sampling with a microvolumetric syringe enabled rapid, accurate removal of the sample. Only 100µl was withdrawn for each sample and replacement of the medium was therefore not necessary. When the samples were re-injected at the end of the initial HPLC run, there was no detectable increase in the amount of drug dissolved, thus demonstrating that direct withdrawal of the samples using the microvolumetric syringe was a valid technique.

The dissolution curves of the pure powder in different dissolution media are depicted in Fig.4.1. while the individual results are listed in Table A4.1. It can be seen that mephenoxalone initially dissolves more slowly in water than in GF or IF; only after 40 minutes were the three dissolution profiles comparable. Mephenoxalone dissolved slightly faster in IF than in GF for the first 10 minutes, but thereafter the profiles were comparable.

The individual results for the dissolution of mephenoxalone from the tablet dosage form in GF are listed in Table A4.2 and the mean dissolution curve for 6 tablets is depicted in Fig.4.2. For comparative purposes, the dissolution curve for the pure powder in GF has been superimposed. It can be seen that, for the first 30 minutes, the release of mephenoxalone from the tablets proceeded more rapidly than the dissolution of the powder; while approximately 88 percent of the stated dose had dissolved from the tablets after 10 minutes, only about 56 percent of the powder had dissolved after the same length of time. However, after half an hour, more than 90 percent of the

FIGURE 4.1 Dissolution profiles for mephenoxalone powder (200 mg) in water, GF and IF

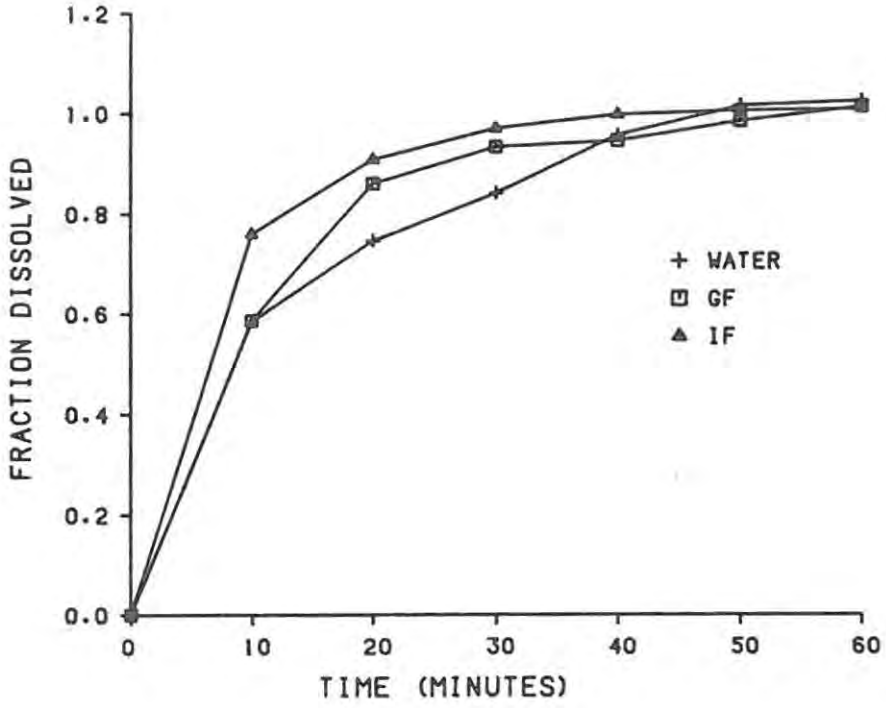
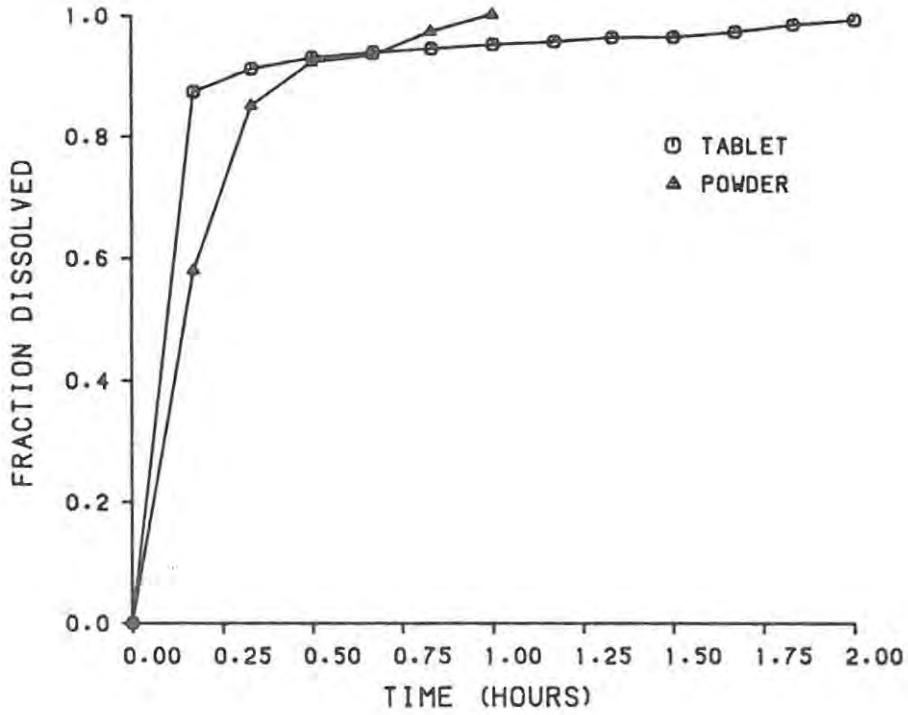


FIGURE 4.2 Dissolution profiles for mephenoxalone powder (200 mg) and a mephenoxalone-containing tablet (200 mg) in GF.



mephenoxalone had dissolved from both the powder and the tablets. The dissolution of mephenoxalone from both dosage forms was essentially complete after 1 hour.

The faster initial rate of *in vitro* dissolution from the tablets has been explained by the manufacturer. In order to overcome any anticipated difficulty with *in vivo* absorption of mephenoxalone, resulting from its poor solubility in water, the tablets were specifically formulated to facilitate a rapid release and dissolution of the drug from the dosage form.

CHAPTER FIVE

CLINICAL TRIALS AND *IN VIVO* ASSESSMENT OF PHARMACOKINETICS OF MEPHENOXALONE

5.1 CLINICAL TRIALS

In vivo trials, using healthy human volunteers were conducted to enable the elucidation of the pharmacokinetic parameters of mephenoxalone after a single oral dose of 400mg powder dispersed in 150ml water. A comparison of the relative bioavailability of a new mephenoxalone-containing tablet (Adcock-Ingram Laboratories Ltd., Johannesburg, South Africa) with that of the pure powdered drug, was also conducted.

5.1.1 Experimental

5.1.1.1 Pilot Trial

A pilot study, using one volunteer, was conducted for the following reasons:

(a) to assess the effectiveness of the analytical method to measuring serum and urine concentrations of mephenoxalone after a single oral dose of 400mg dispersed in water,
(b) to establish the expected serum and urine concentrations of mephenoxalone after the dispersed dosage form, and
(c) to establish the frequency and times of sampling necessary to adequately describe the absorption and disposition of mephenoxalone in terms of pharmacokinetic parameters.

The volunteer was a healthy 20 year old male, weighing 74kg, who was a non-smoker with no previous history of kidney or liver disease and who infrequently partook of alcoholic beverages. Blood and urine samples, collected prior to the trial, were subjected to haematology, blood

chemistry and urinalysis tests (Section 5.1.1.2) and were found to be normal. Prior to admission to the trial, the patient received a typed copy of the trial protocol, and signed a consent form. He received an honorarium for participating in the trial and adhered to the same standard procedures as those described in Section 5.1.1.4.

The patient received a single oral dose of 400mg mephenoxalone powder dispersed in 150ml water followed by a further 100ml water. Blood samples were collected as described in Section 5.1.1.6 at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 and 24 hours after ingestion of the test dose. Urine samples were collected as described in Section 5.1.1.7 during the time intervals 0-2, 2-4, 4-6, 6-10, 10-14 and 14-24 hours. Serum and urine samples were analyzed according to the methods described in Sections 5.1.1.8 and 5.1.1.9, respectively.

5.1.1.2 Six Patient Study

Six young, healthy human volunteers who were non-smokers and infrequent drinkers or abstainers participated in the trial. The demographic data of the volunteers are summarized in Table 5.1. Volunteers were chosen on the basis of physical health and the following haematology, blood chemistry and urinalysis tests:

Haematology:	haemoglobin, haematocrit, WBC, RBC, platelet count, differential count.
Blood chemistry:	serum creatinine, serum urea, blood glucose, serum bilirubin, serum alkaline phosphatase, SGPT and SGOT.
Urinalysis:	specific gravity, bile, pH, protein, glucose, RBC, WBC, epithelial cells, granular casts.

Prior to admission to the trial, each volunteer received a typed copy of the trial protocol and signed a consent form. Volunteers were paid an honorarium for participating in the trial and adhered to the standard procedures described in Section 5.1.1.4.

TABLE 5.1 Details of volunteers

CODE NAME	SEX	AGE	BODY MASS (KG)	HEIGHT (CM)
WA	male	22	63	170
DW	male	21	75	180
MG	male	24	70	181
KZ	female	22	62	165
KR	female	21	59	175
KD	female	19	57	155

5.1.1.3 Treatments

Trial 1: Test dose 1 consisted of a single oral dose of 400mg mephenoxalone powder, dispersed in 150ml of water. Ingestion of this dose at 0 hours was immediately followed by a further 100ml water.

Trial 2: Test dose 2 consisted of a single oral dose of two tablets (each tablet containing a combination of paracetamol, codeine phosphate and 200mg mephenoxalone). This dose was ingested, at 0 hours, with 250ml water.

5.1.1.4 Standardization Procedures

All volunteers had to conform to the following restrictions:

(a) No drugs, including over-the-counter preparations, were allowed for at least a week before the trial and for the duration of the trial.

(b) No alcohol was to be consumed for at least 48 hours before the trial and for the duration of the trial.

(c) No caffeine-containing foods or drinks, including coffee, tea, chocolate and cola drinks, were to be ingested for at least 48 hours before, and for the duration of the trial.

(d) No food or drink was to be ingested for 10 hours before the start of the trial.

The volunteers were allowed to move about freely during the trial but had to refrain from strenuous activities.

A standardized breakfast consisting of toast, with margarine and jam, and 250ml orange juice was served 2 hours after the start of the trial. A standard low fat lunch consisting of chicken, rice and vegetables followed by fresh fruit-salad was given to all the volunteers 3 hours later.

5.1.1.5 Sampling Schedules for the Trials

The sampling schedule used for both Trial 1 and Trial 2 is given in Table 5.2.

5.1.1.6 Collection and Storage of Blood Samples

An indwelling 0.8mm butterfly catheter (21G, Terumo Corporation, Tokyo, Japan) was inserted into a suitable vein in the forearm and securely strapped into position with adhesive tape so as to facilitate complete mobility of the arm. Each 10ml blood sample was withdrawn from the butterfly through a sterile hypodermic needle (0.8mm, Promex (Pty) Ltd., Bergvlei, South Africa) by syringe aspiration. The butterfly was flushed, after sample withdrawl, with 1.5ml sterile saline solution (Keagrams Ltd., Johannesburg, South Africa) containing 50 i.u./ml heparin . Immediately prior to sampling, the butterfly was cleared of the heparin solution by the withdrawal of 2ml of blood which was then discarded, a new syringe and needle being used for withdrawing the 10ml blood sample. The sample was transferred to a clean, labelled Vacutainer tube which was stoppered and allowed to stand for 20 minutes for the blood to clot. The tube was centrifuged at 3000rpm for 10 minutes whereafter the serum was transferred to a clean, labelled Vacutainer tube.

TABLE 5.2 Sampling schedule for Trials 1-2

BLOOD SAMPLING INTERVAL	URINE SAMPLING INTERVAL	TIME
a	b	
0	0	8.00am
10 min		8.10am
20 min		8.20am
30 min		8.30am
40 min		8.40am
50 min		8.50am
1 h		9.00am
1 h 10 min		9.10am
1 h 20 min		9.20am
1 h 30 min		9.30am
1 h 45 min		9.45am
2 h	2 h	10.00am
	BREAKFAST	
2 h 30 min		10.30am
3 h		11.00am
4 h	4 h	12 noon
4 h		1.00pm
	LUNCH	
6 h		2.00pm
8 h	8 h	4.00pm
10 h		6.00pm
12 h		6.00pm
12 h	12 h	8.00pm
	14 h	10.00pm
24 h	24 h	8.00am

a Test dose ingested after collecting a blank

The serum samples, collected at the appropriate sampling times in the manner described above, were stored at -20°C for a maximum of 2 weeks until analysis.

5.1.1.7 Collection and Storage of Urine Samples

The collection of urine samples was controlled by the volunteers themselves and was, therefore, not subject to the same degree of control as the collection of blood samples. The importance of strict adherence to the sampling procedure was stressed in both verbal and written instructions to the volunteers.

The total urine output for each sampling interval was collected in a large measuring cylinder. Volunteers were requested to empty their bladders completely at the end of each interval. The total volume of urine was measured and a representative sample of 10ml was transferred to a clean, labelled Vacutainer tube; the remainder of the urine was discarded. Urine samples were stored at -20°C for a maximum of 1 week until analysis. Urine passed at times other than those specified in the trial protocol was collected as previously described, the volume and the time of voiding being accurately recorded.

5.1.1.8 The Analysis of Serum Samples

Serum samples were brought to room temperature and then mixed on a vortex mixer before being analyzed. The order in which they were analyzed was randomized so as to avoid sequential effects. The samples were prepared for injection according to the method described in Section 3.5.1.3, and 10µl aliquots of the reconstituted residues were injected onto the column (he chromatographic employed conditions are described in Section 3.5.1.2).

The peak height ratios (mephenoxalone/internal standard) were used to determine the concentration of drug in serum from the calibration curve of mephenoxalone in serum (Section 3.5.1.4).

5.1.1.9 The Analysis of Urine Samples

Urine samples were brought to room temperature, mixed using a vortex mixer, and then analyzed according to the method described in Section 3.6.1.3. Ten microlitre samples were injected onto the column (the chromatographic conditions aredescribed in Section 3.6.1.2).

The peak height ratios (mephenoxalone/internal standard) were used to determine the concentration of drug in urine from the calibration curve of mephenoxalone in urine (Section 3.6.1.4).

5.1.2 Results and Discussion

5.1.2.1 Pilot Trial

In the pilot study, the concentrations of mephenoxalone in serum ranged from below 0.1µg/ml up to 7µg/ml over 24 hours. The sensitivity of the analytical procedure was adequate for the purpose of this study. The method of analysis proved to be rapid, accurate and reproducible.

It was found that absorption of the drug was rapid, with the peak blood concentration occuring between 30 and 60 minutes after ingestion of the test dose. It was not possible, however, to determine either the exact time of peak concentration (tmax) or the corresponding concentration (Cmax) because insufficient blood samples were collected during the early phase of the trial. The sampling schedule for subsequent trials was, therefore, rearranged to accommodate 10 minute blood sampling intervals of 10 minutes for the first 1.5 hours and one extra sample at 1.75 hours.

The analysis of urine samples collected in the pilot study indicated that only a small percentage (0.9%) of the administered dose was excreted as unchanged mephenoxalone after 24 hours. In order to confirm this finding, urine samples were collected for the duration of Trial 1.

5.1.2.2 Six Patient Study

Six healthy volunteers, aged 21.5 ± 1.5 years, weighing 64.3 ± 6.3 kg and of average height 171.0 ± 9.0 cm, participated in the clinical study. The volunteers comprised both males (3) and females (3) so as to include both sexes even though the sample sizes were not statistically sufficient. The scale of the study was limited not only by the lack of suitable volunteers but also by the lack of suitable facilities for a large-scale trial. The same volunteers participated in both Trial 1 and Trial 2 but one subject (KD) was unable to participate in Trial 2 which was therefore conducted using only 5 volunteers. Subject KR was admitted to the trials prior to the knowledge that she suffered from a mild case of Gilberts' syndrome. The pathology of this condition is reported in the literature (87). The implications of this disorder on the results of the study are discussed in Section 5.2.2.

Mephenoxalone was well tolerated by the volunteers for the duration of Trial 1. Following the administration of the tablet dosage form (Trial 2), however, two subjects experienced untoward effects which were unrelated to the blood concentration of mephenoxalone; subject KZ reported an onset of nausea at 15 minutes after dosing, while subject KR reported feeling drowsy at 30 minutes after dosing. These effects, however, were mild and transient.

The test dose for Trial 1 should, ideally, have been administered as a solution. Mephenoxalone is, however, insoluble in water (2), and the dose was therefore administered as a dispersion (400mg powder, dispersed in 150ml water). This was immediately followed by a further 100ml water ingested in small amounts to ensure that any powder that may have remained in the mouth was subsequently swallowed. No report was received of either bitterness of the dosage form or manipulative difficulty of the procedure.

The use of an indwelling butterfly catheter for collecting blood samples was found to be convenient. However, in one subject (KZ) the butterfly became blocked as a result of insufficient flushing with heparin. The delay in sampling caused by the removal and subsequent

replacement of the 'butterfly' resulted in the omission of a blood sample scheduled to be drawn at 1 hour 10 minutes. Apart from the above omission, the *in vivo* study followed the prescribed protocol.

The individual concentration-time data for Trials 1 - 2 are listed in Tables A5.1 and A5.2, and the curves are depicted in Fig.5.1. The mean serum concentration profiles after the ingestion of the test doses are shown in Fig.5.2.

Urinary excretion data for Trial 1 are shown in Table A5.3 and plotted in Fig.5.3. After 24 hours, the percentage of the administered dose recovered from urine as unchanged mephenoxalone was 1.17 $\pm$ 0.58 percent. This agrees well with values published for beagle dogs (6). In Trial 1 the recovery of mephenoxalone from urine was not monitored for longer than 24 hours, because urine concentrations of the drug could not be accurately measured after this time. The urine data were excluded from pharmacokinetic analysis because of the low recovery of mephenoxalone.

5.2 PHARMACOKINETIC ANALYSIS OF DATA

The analysis of concentration-time data by pharmacokinetic methods traditionally involves the use of compartment models *e.g.* one compartment, two-compartment and multi-compartment models (88). These classical methods of data analysis are based upon linear processes, and each model is represented mathematically as a linear equation in the form of a sum of coefficients and exponential terms. However, a compartment model characterizes only the rate-limiting steps that occur during a drug-biological system interaction and, as such, it is only an approximation of a biological system. The use of a pharmacokinetic compartment model to describe drug disposition does, however, permit the simulation and prediction of pharmacokinetic data.

In the last decade there has been a move away from the traditional approach towards alternative methods for the pharmacokinetic analysis of drug disposition *e.g.* non-compartmental and model-independent approaches. These have been extensively reported and reviewed in the literature (89, 90, 91). The most popular of these approaches is the

FIGURE 5.1 Individual serum concentration-time profiles after the ingestion of 400 mg mephenoxalone administered as a dispersion (▲) and as tablets (⊙)

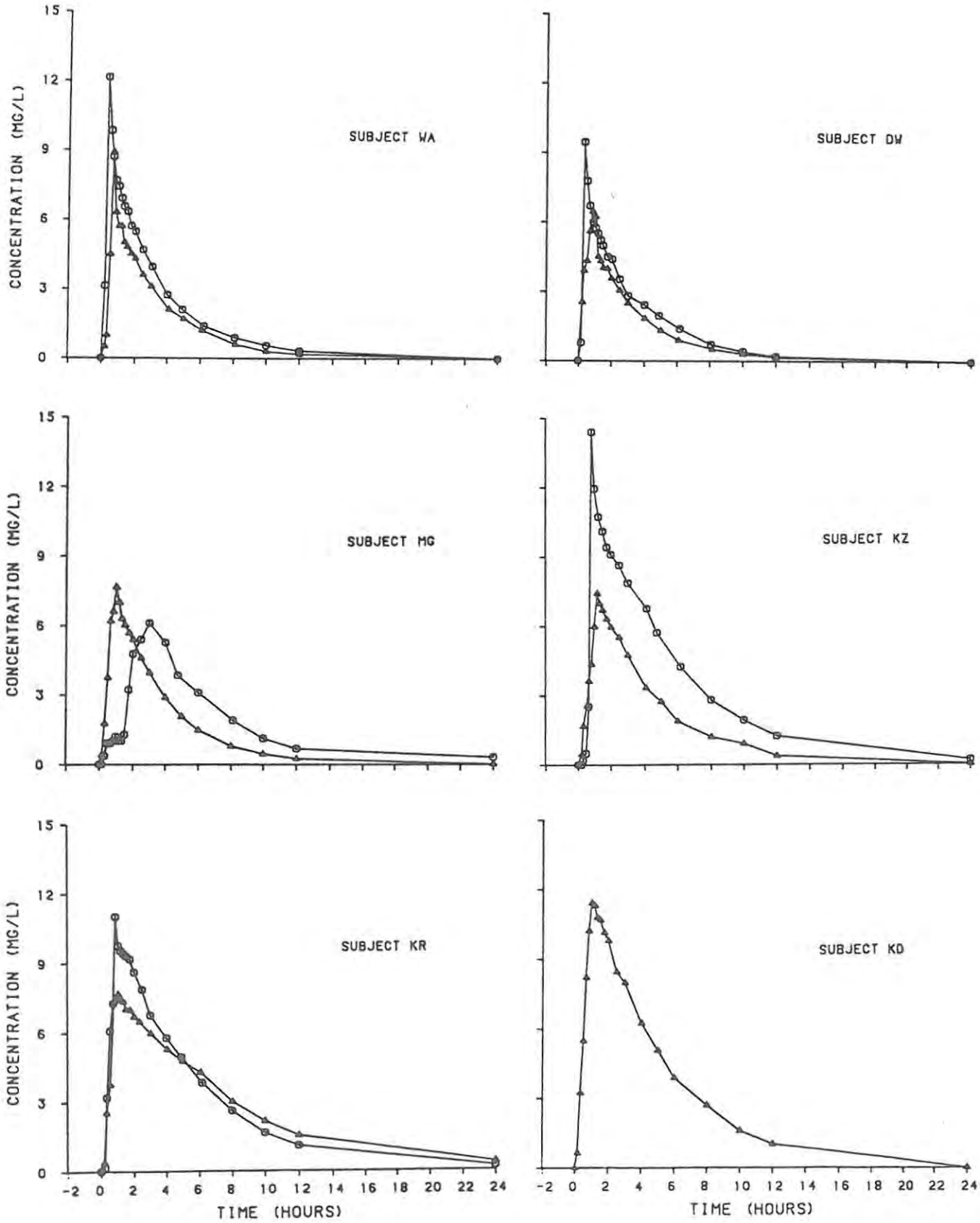


FIGURE 5.2 Mean serum concentration-time profiles after ingestion of 400 mg mephenoxalone administered as (a) a dispersion, and (b) tablets.

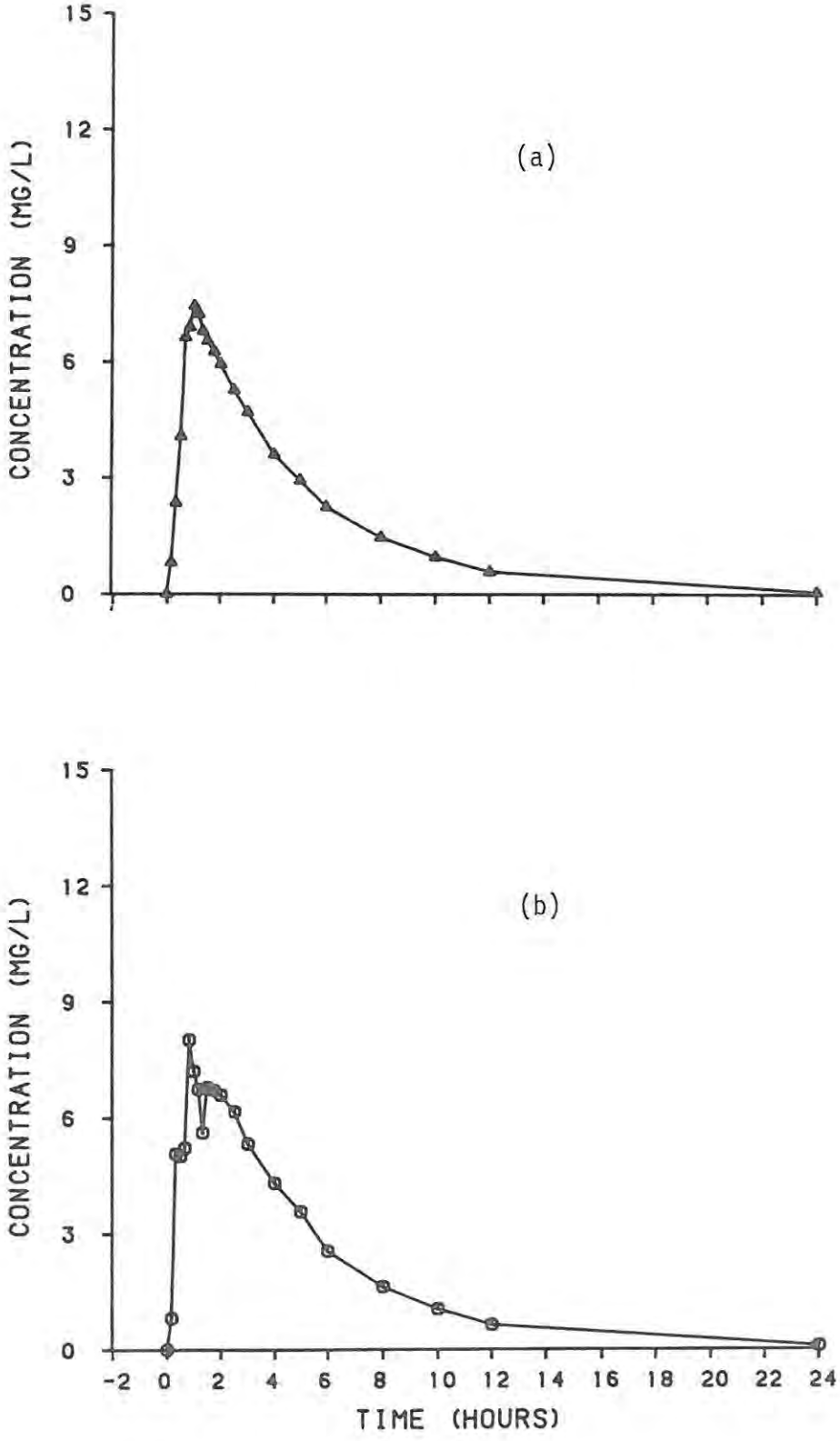
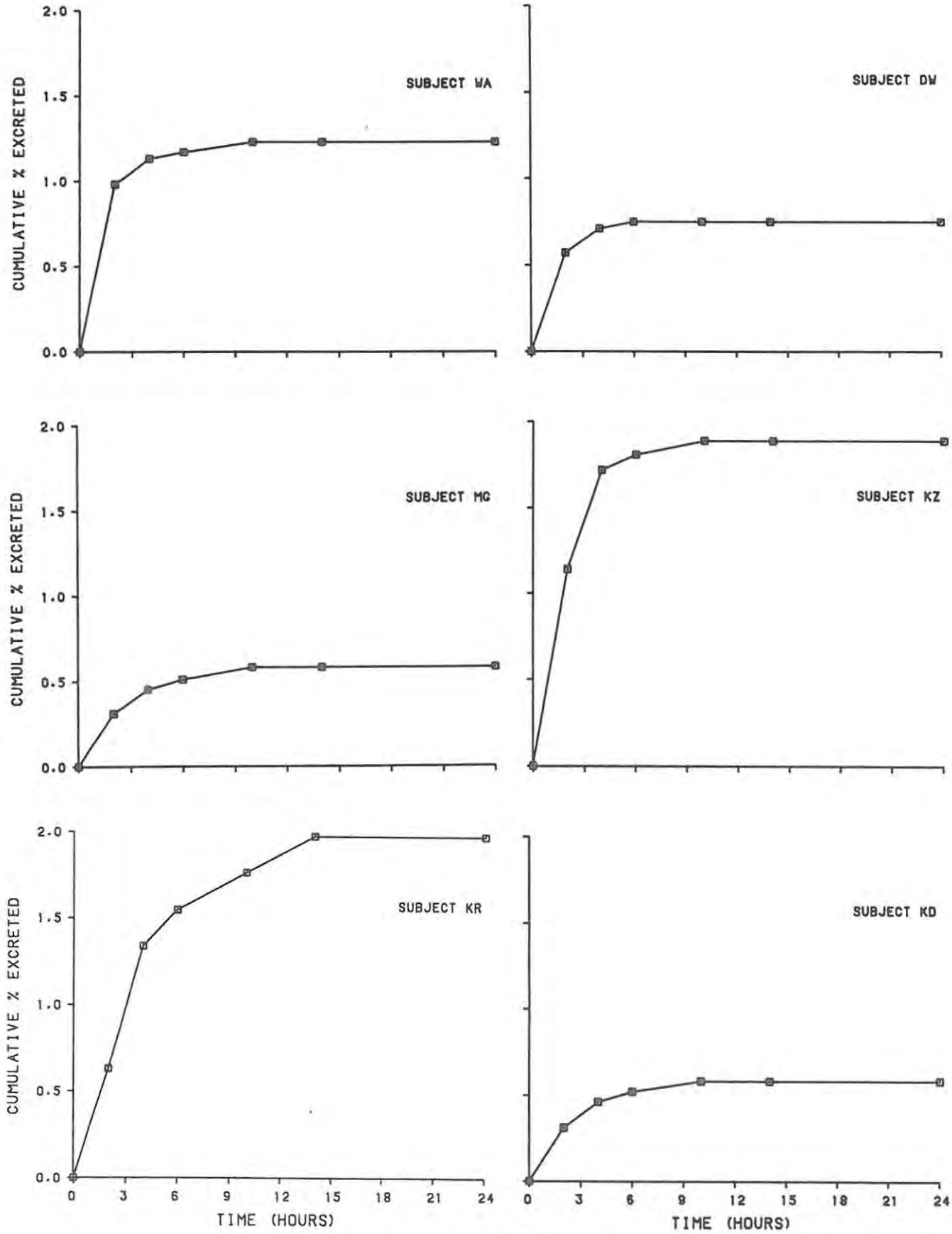


FIGURE 5.3 Plots of cumulative urinary excretion *versus* time after the ingestion of 400 mg mephenoxalone dispersed in water.



model -independent method using statistical moments. The method was first reported in 1978 by Yamaoka *et al.* (89) and Cutler (92), and the concepts, methods of calculation and potential errors in moment analysis have been extensively reviewed by Riegelman and Collier (93). This approach assumes only that all dispositional processes are described by first order kinetics, with elimination occurring from the rapidly equilibrating or central compartment. The advantage of using statistical moments is that they are dependent only on the observed time course data and not on the pharmacokinetic compartment model. This approach has many significant applications, particularly in cases where a drug is administered by a non-instantaneous input (n.i.v.) *e.g.* orally; it allows the estimation of the times involved in the *in vivo* release and absorption processes that occur. Statistical moment analysis has also found widespread application in comparative bioavailability studies.

Bioavailability of a drug is defined as the rate and the extent to which a drug becomes available at the site of action (94). Currently, the most common procedure for assessing and comparing rates of absorption is to use peak plasma concentration (Cmax) and time to peak (tmax); these are, however, only rough estimates containing minimal information about the absorption process (95). Ideally, the rate of absorption should be expressed either numerically or as a mathematical function. The methods that have been developed to obtain an expression for the rate of absorption, in such terms, have been extensively reported and reviewed (89, 90, 92, 93, 96-115). The rate of absorption is commonly expressed in terms of ka, a hybrid constant which is considered to describe the overall rate of appearance of a drug in the plasma. When only oral data are available, the estimation of this parameter is usually performed using either the method of residuals, *i.e.* feathering (88), or the Wagner-Nelson method (97). However, in cases where the drug is extremely rapidly absorbed and few data points are available during the absorption phase, these methods are difficult to apply. An alternative method of characterizing the rate of absorption is by statistical moment analysis (93). The statistical moment that is most frequently used to describe the

absorption process is the mean absorption time (MAT); this is defined as the mean time involved in the *in vivo* release and absorption processes as they occur in the compartment.

Knowledge of the rate of absorption does not, by itself, enable complete characterization of the absorption process; both the rate and the extent of absorption must be known. The extent of absorption is reflected by the fraction, F, of the dose, D, which reaches the general circulation. This parameter is usually estimated by calculating the area under the concentration-time curve (AUC), and it is most popularly calculated by the linear trapezoidal method. However, in the absence of i.v. data this parameter cannot be accurately assessed (94).

In this study, both compartment models and model-independent methods were used for the pharmacokinetic analysis of mephenoxalone concentration-time data.

5.2.1 Methods of Clinical Trial Data Analysis

The experimental data obtained from Trial 1 and Trial 2 were initially treated using model-independent methods. The parameters Cmax and tmax were obtained directly from the individual serum concentration-time data. The areas under the concentration-time curves, from time zero to infinity ($AUC_{0\rightarrow\infty}$) were calculated by the linear trapezoidal method, with extrapolation to infinity. The terminal rate constant, λ_z was calculated by linear regression of the terminal slope of a semilog plot of serum concentration of mephenoxalone *versus* time. From this value, the elimination half-life, $t_{1/2}$, was calculated (Equation 5.1).

$$t_{1/2} = \frac{0.693}{\lambda_z} \qquad \text{Equation 5.1}$$

The clinical trial data were then analyzed by the method of residuals, in an attempt to calculate the values of ka and to assign a possible compartment model to drug behaviour. Assuming a linear one-compartment model with first order absorption, the Wagner-Nelson equation (103) was applied to the serum concentration-time data to

yield absorption plots of fraction absorbed *versus* time. The absorption rates were calculated from the slopes of semilog plots of the fraction of the dose remaining to be absorbed *versus* time.

The nonlinear regression computer program NONLIN (116) was applied to the data in an attempt to fit the experimental data to either a one body-compartment model (1BCM) or a two body-compartment model (2BCM). The set of differential equations that describes these models are listed, together with their exponential functions, in Table 5.3. The DFUNC subroutine of the computer program was modified for each model. The individual data sets were fitted to obtain the least squares estimates of the pharmacokinetic parameters (NONLIN uses the sum of squares, and not the correlation coefficient as the fitting criterion).

Symbols are defined as follows:

C	plasma concentration at time, t, after administration
A, B, E	hybrid intercept terms
α, β	hybrid rate constants
k_a	first order absorption rate constant
k_{10}	first order constant associated with elimination of drug from the central compartment
k_{12}, k_{21}	first order rate constants associated with movement of drug from central compartment to peripheral compartment (k_{12}), and vica-versa (k_{21}).
$\frac{dC_1}{dt}$	rate of change of drug concentration in the central compartment
$\frac{dA_2}{dt}$	rate of change in amount of drug in the peripheral compartment
C_1	concentration in the central compartment
C_2	concentration in the peripheral compartment
D	dose of the drug
V_1	volume of the body

The absorption and disposition of mephenoxalone, after oral dosing, were also evaluated using statistical moment analysis (93). The parameters, mean residence time after oral dosing (MRT_{po}), mean

TABLE 5.3 Differential equations and exponential functions describing a 1BCM and a 2BCM

COMPARTMENT MODEL	DIFFERENTIAL EQUATIONS DESCRIBING THE RATE OF CHANGE OF DRUG	EXPONENTIAL FUNCTION
1BCM 1 k_a, linear elimination	$\frac{dC}{dt} = K_a \frac{De^{-k_a t}}{V} - k_{10}C_1$	$C = Be^{-k_{10}t} - Ee^{-k_a t}$
2BCM 1 k_a, linear elimination	Peripheral compartment $\frac{dA_2}{dt} = K_{12}C_1V_1 - k_{21}A_2$ Central compartment $\frac{dC_1}{dt} = k_a \frac{De^{-k_a t}}{V_1} + k_{21}\frac{A_2}{V_1} - k_{12}C_1 - k_{10}C_1$	$C = Ae^{-\alpha t} + Be^{-\beta t} - Ee^{-k_a t}$

absorption time (MAT) and mean *(in vivo)* dissolution time (MDT) were calculated as follows:

$$MRT_{po} = \frac{AUMC_{\infty}}{AUC_{\infty}} \qquad \text{Equation 5.2}$$

where $AUMC_{\infty}$ is the area under the first moment of the plasma curve, and

AUC_{∞} is the area under the concentration-time curve

$$MAT = MRT_{po} - MRT_{iv} \qquad \text{Equation 5.3}$$

where MRT_{iv} is the mean residence time after an i.v. dose

Since i.v. data were not available in the studies, MRT_{iv} was estimated indirectly from the oral data, according to the method of Riegelman and Collier (93). The disposition of mephenoxalone was generally described by a mono-exponential decline, and MRT_{iv} was therefore determined from Equation 5.4.

$$MRT_{iv} = \frac{1}{\lambda_z} \qquad \text{Equation 5.4}$$

Calculation of the mean *(in vivo)* dissolution time for a solid dosage form (Equation 5.5) enables the contribution of the dissolution process to be separated from the apparent absorption and disposition processes.

$$MDT_{prod} = MAT_{prod,\ uncorr} - MAT_{soln,\ uncorr} \qquad \text{Equation 5.5}$$

Where $MAT_{prod,\ uncorr}$ and $MAT_{soln,uncorr}$ are the uncorrected mean absorption times for the product and for the solution of the drug, respectively.

However, as a result of the poor water solubility of mephenoxalone, solution data were not available in this investigation. Equation 5.5

was therefore adapted for dispersion studies (Equation 5.6). Although this is not ideal, it does provide an estimate of the *in vivo* dissolution time of the tablet dosage form relative to that of the dispersed dosage form.

$$MDT_{tablet} = MAT_{tablet} - MAT_{dispersion} \qquad \text{Equation 5.6}$$

In cases where a negative result is obtained, it can be assumed that the *in vivo* dissolution of mephenoxalone proceeds more rapidly from the tablets than from the dispersion.

5.2.2 Results and Discussion

The values of Cmax, tmax, AUC_∞, λ_z and t1/2, obtained from the serum data for Trial 1 and Trial 2, are presented in Table 5.4.

In the 400mg single dose dispersion study (Trial 1), the absorption of mephenoxalone was rapid, with peak blood concentrations occurring 40-70 minutes (57 $\pm$ 10 minutes) after ingestion of the dose. Peak concentrations ranged between 6.46 and 11.42µg/ml (8.24 $\pm$ 1.58µg/ml). The AUC_∞ values for two of the subjects (KR, KD) were almost double the AUC_∞ values for the other 4 subjects, suggesting that KR and KD either absorbed the administered dose to a greater extent or that they eliminated the parent drug more slowly than did the other subjects. Although the elimination half-life for KD was slightly longer than the average elimination half-life for the other four subjects, it is unlikely that this was the sole factor responsible for the high AUC_∞ value. Conversely, the elimination half-life for KR was significantly longer than the other t1/2 values, indicating that a slow rate of elimination may, indeed, be the major factor responsible for the high AUC_∞ value in this case. This subject had a large t1/2 value and a high AUC_∞ value in both Trial 1 and Trial 2.

TABLE 5.4 Bioavailability parameters for mephenoxalone dispersion and tablet data.

TEST DOSE	SUBJECT	PARAMETERS				
		t_{max} (hrs)	C_{max} (µg/ml)	AUC_{∞} (µg/ml.hr)	λ_z (hr^{-1})	$t_{1/2}$ (hrs)
Mephenoxalone Dispersion (400mg)	WA	0.67	8.85	22.30	0.312	2.22
	DW	0.83	6.46	19.49	0.314	2.21
	MG	1.00	7.66	27.00	0.312	2.22
	KZ	1.17	7.41	31.82	0.264	2.63
	KR	1.00	7.65	59.38	0.147	4.71
	KD	1.00	11.42	59.97	0.224	3.09
	MEAN	0.95	8.24	36.66	0.262	2.85
	(SD)	(0.16)	(1.58)	(16.72)	(0.061)	(0.89)
Mephenoxalone -containing Tablets (2 x 200mg)	WA	0.33	12.14	31.56	0.284	2.44
	DW	0.50	7.77	25.12	0.297	2.33
	MG	2.50	6.10	26.29	0.309	2.24
	K2	0.83	14.39	63.49	0.180	3.85
	KR	0.83	11.00	58.35	0.177	3.92
	MEAN	1.00	10.28	40.96	0.249	2.96
	(SD)	(0.78)	(3.00)	(16.52)	(0.058)	(0.76)
	MEAN excluding subject MG	0.70	11.33	44.63	0.235	3.14
	(SD)	(0.25)	(2.39)	(16.55)	(0.056)	(0.75)

Mephenoxalone is reported to be eliminated primarily by hepatic biotransformation; the major metabolites are the glucuronic acid conjugated forms of two hydroxylated derivatives (6). It may therefore be postulated that subject KR had a long elimination half-life as a result of either a slow rate of conjugation or a slow rate of excretion of the biotransformation products. It is more likely that the former applies because the subject suffered from an hereditary pathological disorder, Gilberts' syndrome, which is characterized by reduced levels of glucuronyl transferase in the liver.

In the 400mg single dose tablet study (Trial 2), peak serum concentrations occurred 20-50 minutes after ingestion of the dose in all but one of the subjects. The peak concentration for subject MG, occurred at 2.5 hours and was of a much lower concentration (6.10µg/ml) than the Cmax values for the other subjects (11.33 $\pm$ 2.39µg/ml). This discrepancy cannot be easily explained as the same restrictions were practised prior to both trials. However, the trials were conducted 4 months apart and changes in the physical state of the subject could have taken place. The slower rate of absorption from the tablets could also be attributed to the effect of codeine phosphate (which was included in the dosage form) on the gastro-intestinal tract (GIT). Codeine is known to cause an increase in the muscle tone of the stomach and of the small and large intestines; this delays both gastric emptying and the passage of contents through the GIT (117).

Excluding the results of MG, it was found that mephenoxalone was absorbed more rapidly from the tablet dosage form than from the dispersion; peak concentrations of mephenoxalone were reached 10-20 minutes earlier and ranged from 17 to 94 percent higher. A possible explanation for this phenomenon is that the dissolution of mephenoxalone from the tablet dosage form is superior to the dissolution from the dispersed dosage form dose. The results obtained in the *in vitro* dissolution testing of the dosage forms (Section 4.3) support the *in vivo* results.

The individual AUC_{∞} values for each trial were compared in order to ascertain the relative bioavailability of the tablet dosage form with respect to the dispersed dosage form. Since it was not possible to determine the absolute systemic bioavailability of mephenoxalone because of the lack of i.v. data, the relative bioavailability, Frel, was calculated from the ratios of the AUC_{∞} values. The results are presented in Table 5.5.

From the mean data, it appears that the tablet dosage form has a greater bioavailability, in terms of Frel, than the dispersed dosage form. However, the individual Frel values show that this was not true for two of the subjects (MG, KR), both of whom absorbed mephenoxalone to a similar extent from both dosage forms. The Frel results for WA and DW are not easy to explain because the elimination half-lives of these subjects were of a similar magnitude for both of the trials. Subject KZ showed a significant increase in the extent of bioavailability for Trial 2 compared with Trial 1. However, the subject disclosed that in the period between trials she had severely restricted her dietary intake, which may have resulted in a decrease in metabolic rate; this would explain both the increase in the elimination half-life and the high AUC_{∞} value for KZ.

Table 5.5 Relative bioavailability of tablet dosage form with respect to dispersed dosage form

	SUBJECT					MEAN (SD)
	WA	DW	MG	KZ	KR	
Frel	1.41	1.29	0.97	1.99	0.97	1.33 (0.38)

From the semilogarithmic plots of the individual concentration-time data (Fig.5.4), it was observed that the declines in mephenoxalone concentrations during the post-absorptive phases were generally mono-exponential processes; biphasic elimination processes were observed in 2 cases: WS (Trial 2), DW (Trial 2), but the initial phases were of extremely short duration. The terminal elimination rate constants were easy to determine because of the abundance of samples taken during the post-absorptive phase. However, very few data points were generally available during the absorption phases and it was therefore difficult to determine the values of the absorption rate constant, ka. However, in cases where 2 or more reliable data points were available, the ka values were estimated using the method of residuals. The results are reported in Table 5.6, with the number of data points shown in parentheses. The ka values which were calculated using the Wagner-Nelson method (103) are also shown in Table 5.6, as are the predicted values using NONLIN (including those cases where values could not be calculated using either the method of residuals or the Wagner-Nelson method). From these results, it appears that WA and DW absorbed mephenoxalone more rapidly from the tablet dosage form than from the dispersed dosage form. This finding correlates well with the values of Cmax and tmax for these subjects. Using the method of residuals and the Wagner-Nelson method, two ka values were calculated for MG in both trials; an initial slow rate of absorption was followed by a faster rate. This effect could not be explained. The ka values obtained for KZ indicate that mephenoxalone is absorbed more slowly from the tablets than from the dispersion; although this may be true in the early stages of absorption, peak concentrations were reached more rapidly and were higher for the tablet dosage form. It can also be seen that large variations exist in the ka values which are calculated using the different methods. These findings demonstrate the inadequacy of using ka to describe the absorption of mephenoxalone after oral dosing.

In contrast to the findings for ka, the results from statistical moment analysis of the data have a good correlation with the Cmax and tmax data. The statistical moment parameters for Trial 1 and Trial 2 are shown in Table 5.7. The mean MAT values indicate that mephenoxalone is rapidly absorbed from both the dispersion and the

FIGURE 5.4 Semilogarithmic plots of serum concentration *versus* time after the ingestion of 400 mg mephenoxalone administered as (▲) a dispersion, and (⊙) tablets.

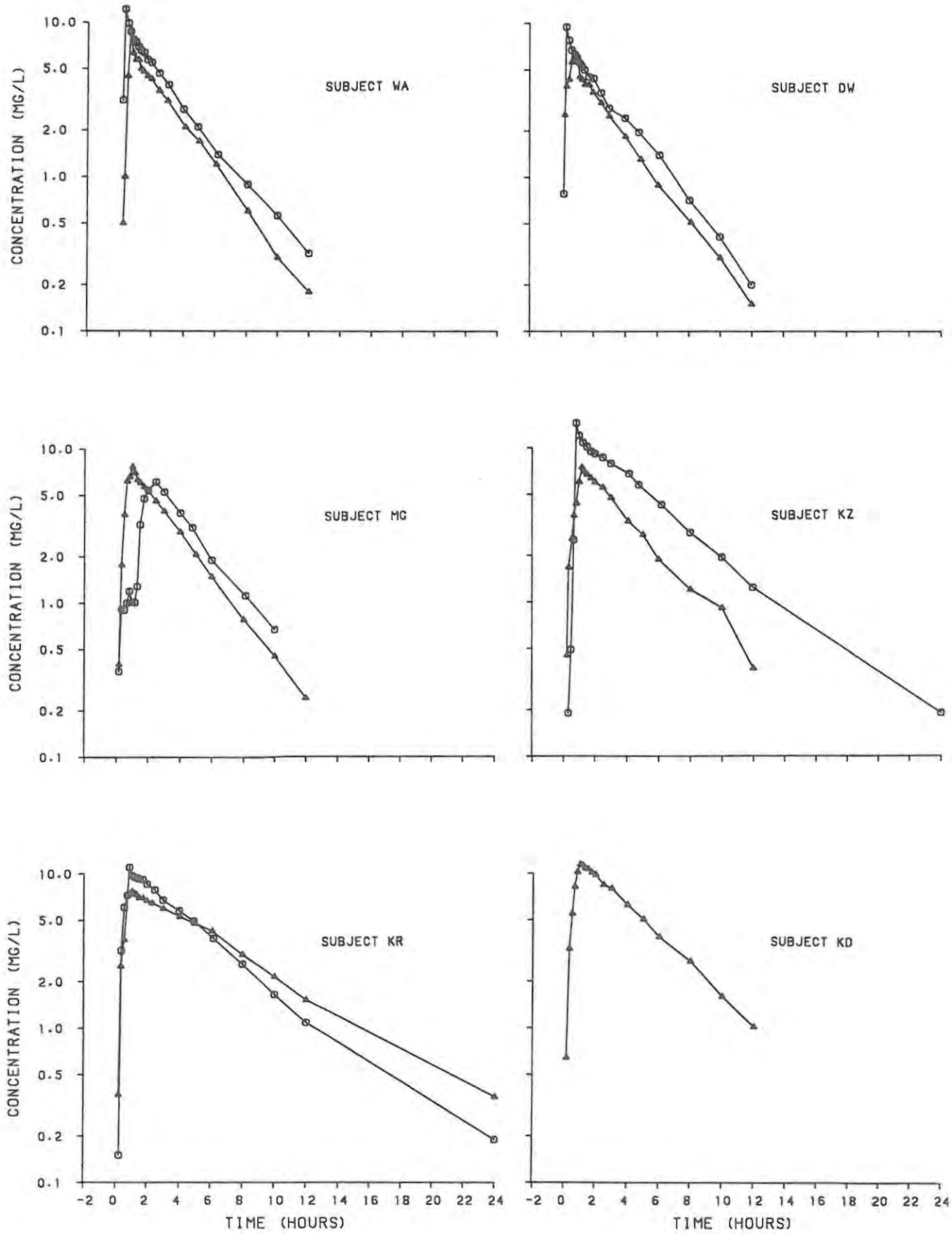


TABLE 5.6 Values of ka(h-1) calculated using different methods

SUBJECT	METHOD OF RESIDUALS	WAGNER-NELSON	PREDICTED VALUES USING NONLIN	
			1BCM	2BCM
DISPERSION STUDY (TRIAL 1)				
WA	1.091 (2)	0.499 (2)	2.152	1.475
DW	- (1)	3.177 (3)	3.000	2.314
MG	1.437 (2) 5.040 (3)	1.266 (2) 4.509 (3)	1.307	1.231
KZ	1.293 (4)	1.157 (2)	1.053	0.679
KR	1.273 (4)	1.082 (4)	1.452	1.347
KD	1.637 (3)	1.549 (3)	1.211	1.098
MEAN (SD)	1.346 (0.182)[a]	1.445 (0.811)[a]	1.696 (0.679)	1.357 (0.495)
TABLET STUDY (TRIAL 2)				
WA	- (1)	- (1)	5.930	4.708
DW	- (1)	- (1)	4.777	3.459
MG	0.433 (8) 2.180 (3)	0.168 (7) 2.022 (3)	0.500	0.436
KZ	0.324 (3)	0.331 (3)	1.145	0.695
KR	3.093 (3)	3.112 (3)	1.295	1.252
MEAN (SD)	1.283 (1.280)[a]	1.204 (1.351)[a]	2.729 (2.190)	2.100 (1.680)

[a] Mean values were calculated by excluding the second ka value for subject MG

tablets, with a more rapid absorption occurring from the latter. After ingestion of the tablets, subject MG displayed a significantly longer MAT than the other subjects.

TABLE 5.7 Parameters obtained from statistical moment analysis of trial data

SUBJECT	PARAMETER				
	MRT (hours)		MAT (hours)		MDTTablet
	Dispersion	Tablet	Dispersion	Tablet	
WA	3.58	3.54	0.38	0.02	-0.36
DW	3.38	3.56	0.20	0.19	-0.01
MG	3.71	4.78	0.50	1.15	0.65
KZ	4.50	5.99	0.71	0.44	-0.27
KR	7.76	5.98	0.96	0.33	-0.63
KD	5.04	-	0.58	-	-
MEAN (SD)	4.66 (1.50)	4.77 (1.09)	0.55 (0.24)	0.43 (0.39)	-0.12 (0.43)

The mean (*in vivo*) dissolution time for the tablet dosage form was, on average, a negative value, which shows that mephenoxalone dissolves more rapidly from the tablets than from the dispersion. An explanation for this phenomenon is provided in Section 4.3. From the values of the transit time for mephenoxalone through the body (MRT po), it can be seen that the mean values did not differ to any significant extent. However, within the individual values there were large inter-trial and intra-trial variations. The long transit times found for KR have already been explained in terms of her pathological disorder. It was, however, interesting to note that the MRT value obtained for the tablet study was shorter than the value obtained for the dispersion study. The tablets contained not only mephenoxalone but also paracetamol and codeine phosphate; both paracetamol (118) and codeine

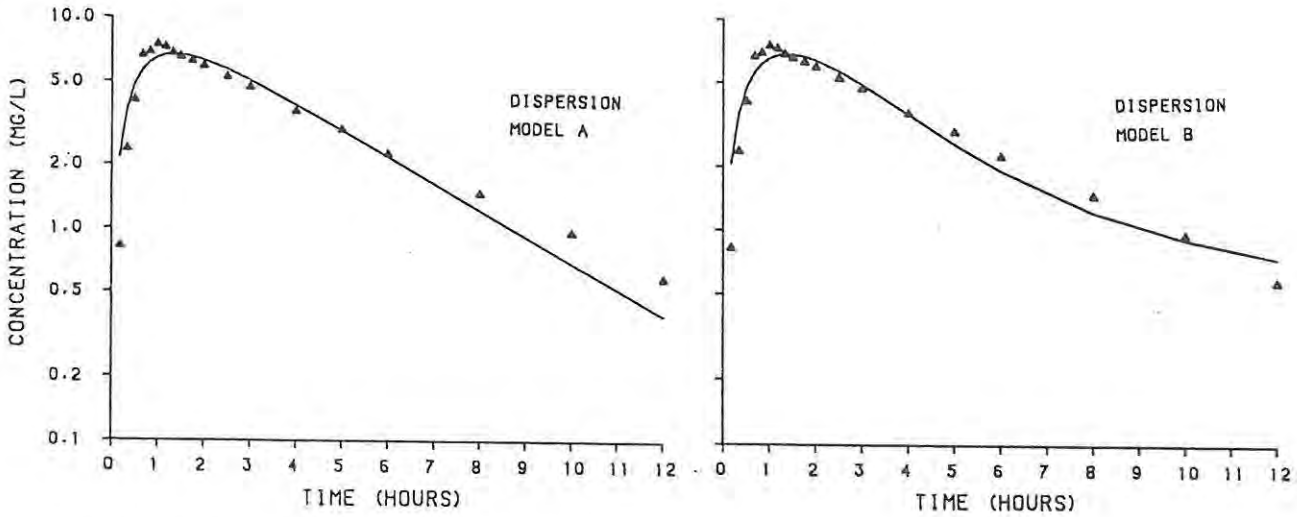
phosphate (119) are extensively metabolized in the liver, with the major metabolites being their respective glucuronide derivatives. Administration of the tablets to a patient with reduced levels of glucuronyl transferase (*i.e.* KR) could lead to saturation of the conjugation processes. This was not, however, found to be the case for KR who, conversely, showed a shorter elimination half-life and, consequently, a shorter transit time for the tablet study. This discrepancy can only be explained by individual variability.

The longer transit time for KZ in Trial 2 can be partly explained by a longer elimination half-life. Subject MG also displayed a longer MRT value in the tablet study than in the dispersion study. This cannot be attributed to a longer elimination half-life because the t1/2 values for Trial 1 and Trial 2 were of a similar magnitude. However, this subject absorbed mephenoxalone very slowly from the tablet dosage form and this may have caused an increase in the mean transit time of the drug through the body. A reduction in gastro-intestinal motility (effect of codeine) could also have contributed to a longer transit time for mephenoxalone after tablet ingestion.

Computer fitting of the data using NONLIN was performed by constraining the value of the terminal elimination rate constant, while the other parameters were altered to try and find fits for each set of data. The serum data sets were fitted to both a 1BCM (Model A) and a 2BCM (Model B). The observed and predicted values for the individual data sets, resulting from fits to both models, are listed in Tables A5.4 - A5.7, while the predicted parameter values are shown in Table A5.8. The observed values and predicted fits to the models for the mean data are graphically presented in Figs.5.5. The values of the correlation coefficient, r, used as an indication of the goodness of fit, for the mean dispersion data, were 0.969 and 0.971 for Model A and Model B, respectively. For the mean tablet data, a correlation coefficient of 0.961 was obtained for both models. Although these figures are not indivative of poor fits, the absorption phase was poorly characterized, with an initial large over-estimation of concentration followed by an inability to attain the high peak concentrations. There was a more rounded peak and a longer time to reach maximum concentration. These effects are more clearly seen from

FIGURE 5.5 Observed values (▲,⊙) and predicted fits (-) to the mean serum data after the ingestion of (a) a dispersion of mephenoxalone, and (b) mephenoxalone-containing tablets, using Model A and Model B.

(a)



(b)

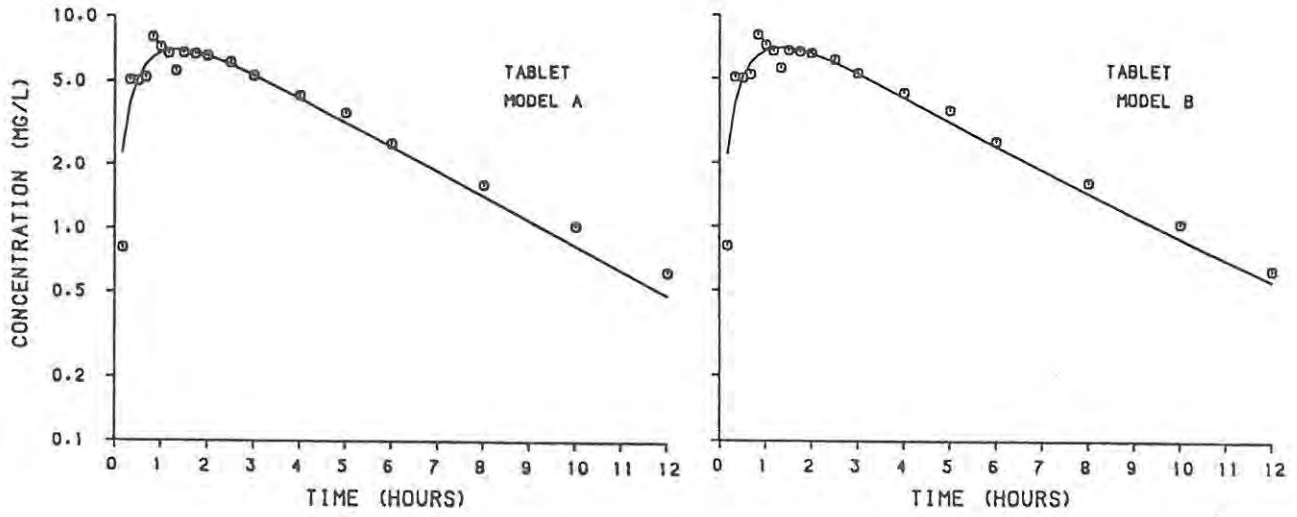
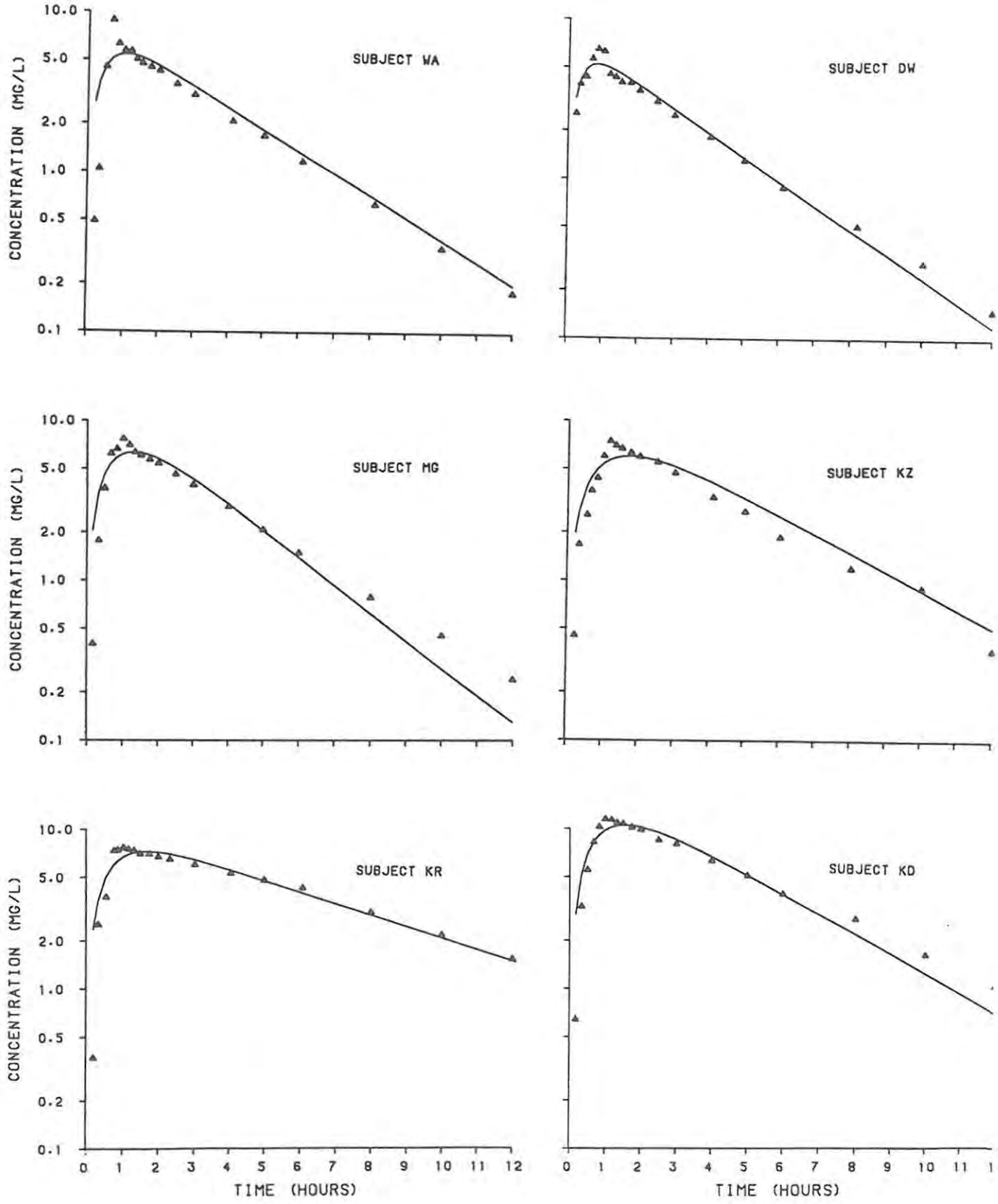
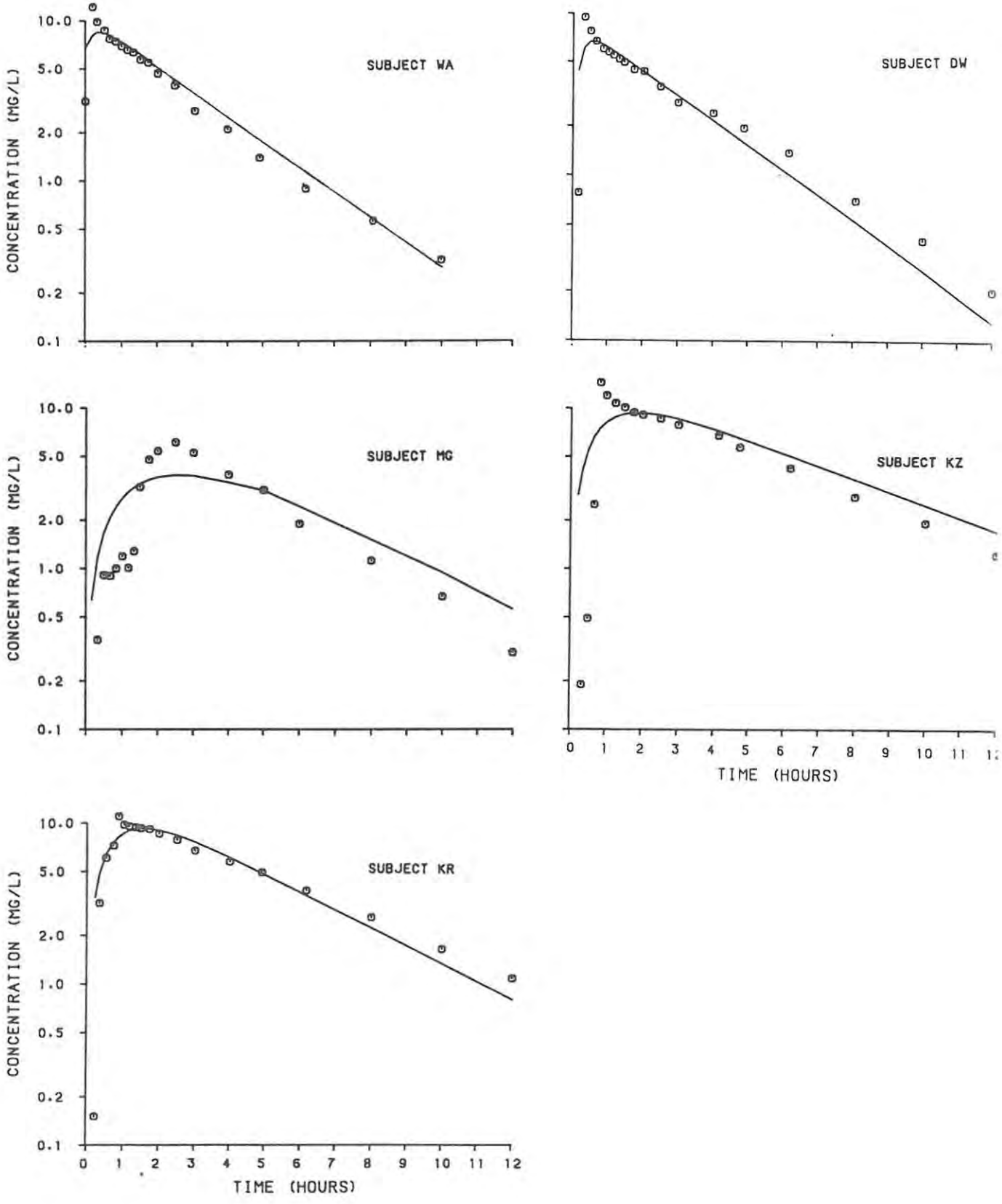


FIGURE 5.6 Observed values (▲,○) and predicted fits (-) to the individual serum data after the ingestion of (a) a dispersion of mephenoxalone, and (b) mephenoxalone-containing tablets, using Model A.

(a)



(b)


SUBJECT WA
SUBJECT DW
SUBJECT MG
SUBJECT KZ
SUBJECT KR
CONCENTRATION (MG/L)
10.0
5.0
2.0
1.0
0.5
0.2
0.1
0 1 2 3 4 5 6 7 8 9 10 11 12
TIME (HOURS)

the fits for the individual data sets (Fig.5.6). Since the fits to Model A and Model B were extremely similar, only the fit to Model A were plotted. The predicted fits of the data in the terminal phases were generally good, but the predicted fits in the absorption phases were poor, thus showing the inadequacy of using a single ka value to describe the absorption process. The use of a model which can accommodate two or more absorption rate constants may produce better fits, but was not investigated in this study.

The difficulties experienced in characterizing the fast absorption phase are not unique to mephenoxalone. Numerous studies have been reported on drugs which exhibit extremely rapid absorption profiles after oral administration (120-125). In most cases the data were fitted to compartmental models in order to obtain an estimate for the absorption rate constant, ka (120-123). Only one of these studies reported using the "stripping" method of Wagner to determine ka (12). Siefert *et al* (125) conducted a study of the pharmacokinetics of ciprofloxacin in rats and monkeys and characterized absorption as the time needed for an increase in plasma concentration from 25 to 75 percent of the maximum concentration. In a clinical study to evaluate the pharmacokinetic profile of pyrazinamide, Bareggi *et al.* (122) analyzed to plasma concentration-time date by computer fitting of the experimental values to various compartment models. However, absorption of the drug was found to be extremely rapid such that in several subjects computing the rate of absorption was impossible, and only Cmax and tmax data were therefore used. In all of the above-mentioned studies, Cmax and tmax data were used to express the absorption profiles and in this study, these parameters together with statistical moment parameters provided the best means of assessing mephenoxalone absorption.

CHAPTER SIX

THERMOSPRAY HPLC-MS OF MEPHENOXALONE AND ITS METABOLITES

6.1 INTRODUCTION

The combination of high-performance liquid chromatography (HPLC) with mass spectrometry (MS) provides a powerful analytical method, combining the benefits of two well-established and complementary techniques. Liquid chromatography (LC) achieves separation of the sample into its components and the mass spectrometer, unlike the more common HPLC detectors *e.g.* UV detectors, can be operated either as a universal detector or as a very selective detector (126-129).

The LC-MS systems adopted in the past have been mainly off-line systems. These involve a non-continuous process in which an intermediate sample-collection step is required prior to MS analysis. Off-line methods have, however, been largely superseded by on-line techniques in which the LC eluent is transferred directly to the mass spectrometer via an interfacing device. The direct coupling of the two systems offers a more convenient means of analyzing multi-component mixtures; there is a reduced danger of sample losses, a more precise quantitative analysis is achieved, and partially resolved chromatographic peaks can be deconvoluted by taking advantage of the high selectivity of the mass spectrometer (128, 130).

On-line LC-MS operation, however, involves the coupling of two highly incompatible disciplines; mass spectrometry requires both a high vacuum and the ionization of molecular species in the gas phase, whereas LC operates under high pressure and is intended for the analysis of substances not volatile enough to be handled by gas chromatography (GC). The developments and approaches aimed at overcoming the difficulties of interfacing LC and MS have been amply reviewed (127, 130, 131). The interfaces which are commercially available include mechanical transport devices and direct introduction methods, both of which have been successfully applied to a wide range of analyses. The moving belt is the most popular mechanical transport device while direct introduction methods such as the diaphragm-type

interface (commonly called direct liquid introduction), electrospray and thermospray are also widely employed.

The progress made in overcoming the difficulty of coupling LC to MS has been accompanied by the development of new, more gentle ionization techniques which produce ions characteristic of the molecular mass (M) (132). These ionization modes include field desorption (133), laser desorption (134), secondary ion mass spectrometry (135), electrohydrodynamic ionization (136), fast atom bombardment (137), liquid ion evaporation (138) and thermospray (139). The majority of these are, however, used exclusively in off-line LC-MS systems. An exception is thermospray (TSP) ionization which was principally designed as an on-line system by Vestal and co-workers (139-142). The advantages of this technique over other coupling methods include its ability to handle conventional LC flow rates, mild ionization conditions, the capacity to use volatile ionic modifiers and high percentages of water in LC solvents, and the ease with which the interface can be adapted to fit existing GC-MS instruments. Most other LC-MS interfaces cannot accommodate either conventional HPLC flow rates or eluents with a high water content. The mechanism of TSP ionization has been investigated but is not yet fully understood (140, 143-146).

The versatility of the TSP technique is reflected not only by its potential for analyzing polar, non-volatile and thermally labile compounds but also by its suitability for the determination of volatile compounds (147), neutral analytes (146) and for *in situ* degradation studies (148). TSP HPLC-MS has been applied to the qualitative and quantitative analysis of numerous compounds of environmental, biological and pharmaceutical importance including dyes (149), pesticides and herbicides (150, 151), mycotoxins (152), peptides (153), phospholipids (154), glucuronides (129), β-lactam antibiotics (148), several drugs and their metabolites in biological fluids (155), and other compounds of pharmaceutical interest (156).

The role of LC-MS in the analysis of pharmaceuticals has been identified by Erni (157). Table 6.1 depicts the areas of application that were highlighted in the report; the primary goals of the technique are to obtain structural information, to identify substances

and to enable the selective, sensitive detection of compounds of interest.

TABLE 6.1 Applications of LC-MS in the pharmaceutical industry

By-products	identification
	quantification of trace compounds
Degradation products	of drug substances in the dosage form
	quantification of special compounds
Bio-assays	plasma
	urine
	other biological materials
Metabolism	identification
	stable isotopes
Screening	

The application of TSP HPLC-MS to the identification of mephenoxalone metabolites is presented below. A review of thermospray theory precedes the experimental study.

6.2 THEORY OF THERMOSPRAY

6.2.1 Mechanism of Thermospray Ionization

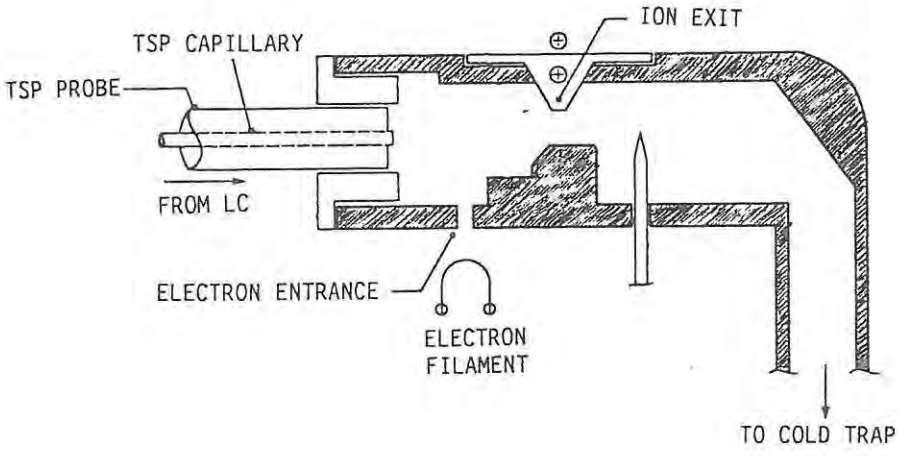
Thermospray is defined as the controlled, partial or complete vaporization of a liquid as it flows through a heated capillary (139, 140). The capillary is located in a probe which serves as a vehicle for introducing the sample into the ion source of the mass spectrometer. A schematic diagram of a typical TSP interface and ion source is shown in Fig.6.1.

TSP ionization of a compound may occur in two different ways, namely, direct (filament-off) ionization and external (filament-on) ionization (139). Both require the presence of an electrolyte in the HPLC mobile phase. The term "direct" ionization refers to the

generation of ions directly from the buffered mobile phase without the assistance of an external source of energy (140, 143, 146). As the LC effluent flows into the heated capillary, a high proportion is vaporized to produce a superheated mist carried in a jet of vapour. Non-volatile molecules are preferentially retained in the mist droplets and in the presence of the electrolyte, the statistical distribution of charge ensures that each droplet carries a slight excess of positive or negative charge. The charge density increases as the droplets continue to vaporize and shrink and sample molecules held in these droplets are often ionized (as in regular chemical ionization). The electric fields generated by the charge on the droplets are sufficient to cause evaporation of sample ions together with a few solvent molecules. Ion-molecule reactions may subsequently occur in the gas phase if exothermic channels are available. Finally, the ions are transported to the mass analyzer and a spectrum is generated.

FIGURE 6.1 Thermospray ion source

Adapted from Goodley P.C. (145).



The filament-on mode utilizes a source of high energy electrons to promote the formation of chemical ionization reagent ions from the solvent molecules (140, 143-145). These, in turn, ionize the sample molecules. The method of ionization chosen for an analysis depends on the sample and on the type of information required (145). The direct ionization mode appears to be applicable only to polar compounds or to pre-formed ions in solution. For non-polar compounds and most medium polarity substances, an external source of electrons is necessary to produce sample-derived ions. Each mode provides a variant of the chemical ionization process but much less energy is transferred to the ions in the direct mode as compared to the filament-on technique (144). Both processes result in the production of very few fragment ions of the analyte molecules; the ammoniated or protonated molecular adduct ions are the most common products (150).

6.2.2 Optimization of TSP Operating Parameters

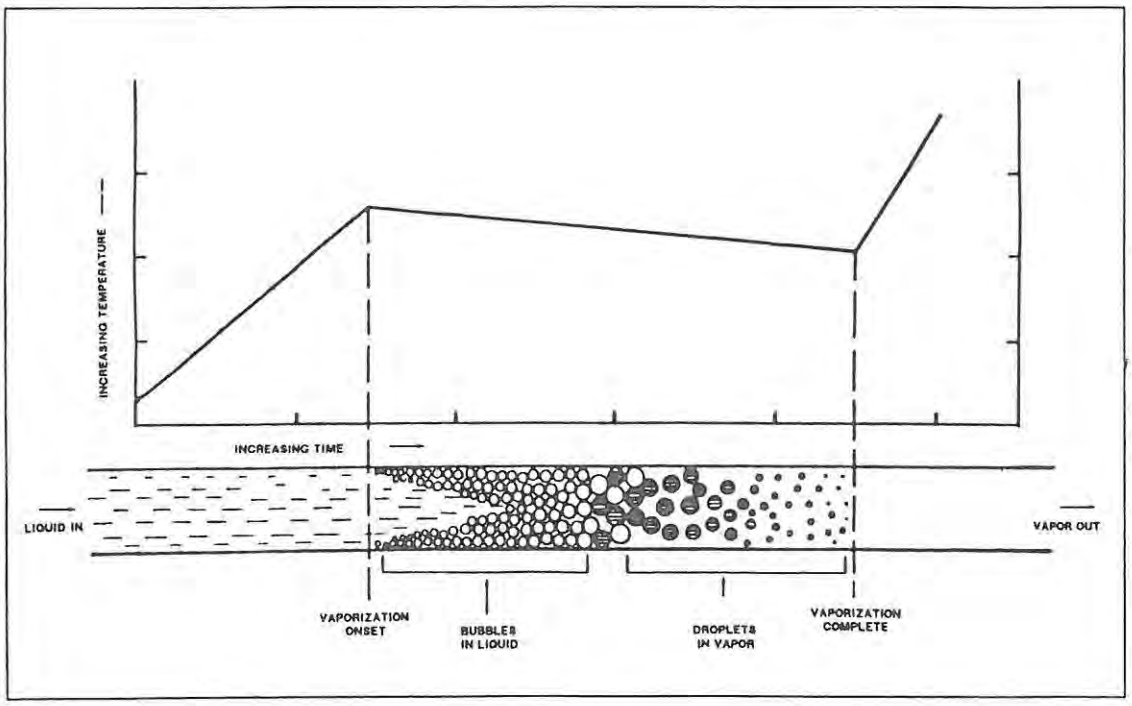
The sensitivity of an analysis and the quality of the spectra obtained by TSP HPLC-MS have been demonstrated to be dependent on several operating parameters, the most important being vaporizer probe temperature, solvent composition and the use of a high energy filament (145, 150, 158). Optimization of these factors will greatly enhance the sensitivity of the TSP technique and extend its range of application.

6.2.2.1 Vaporizer Probe Temperature

The TSP ionization process is dependent on temperature; maximum analyte ion abundance is always obtained at a temperature which allows almost complete vaporization of the LC effluent as it flows through the probe. Optimum probe temperature can be ascertained by various methods (144, 145, 150). By measuring sample ion abundance at different probe temperatures, an optimum response temperature can be determined. Alternatively, solvent ions, rather than solute ions, can be used in a similar manner. This is because all ions have a maximum abundance in the same narrow temperature range. A third method is based on measuring the temperature change of the LC eluate at the probe exit as the probe stem is progressively heated. Figure 6.2

depicts the temperature change as the LC effluent flows through the capillary. A computer program is utilized to generate a hard copy plot of the vaporizer tip temperature *versus* the stem temperature, as depicted in Fig.6.3(b). The plot is called a probe survey scan or vaporizer plot. From this, the temperature at which the liquid/vapour mixture at the probe exit changes to 100% vapour can be ascertained. The phase change is indicated by a steep change in the slope of the plot, referred to as the inflection point. A stem temperature of 90 to 95% of the inflection point value gives the greatest sensitivity in terms of ion abundance, as shown in Fig.6.3(a).

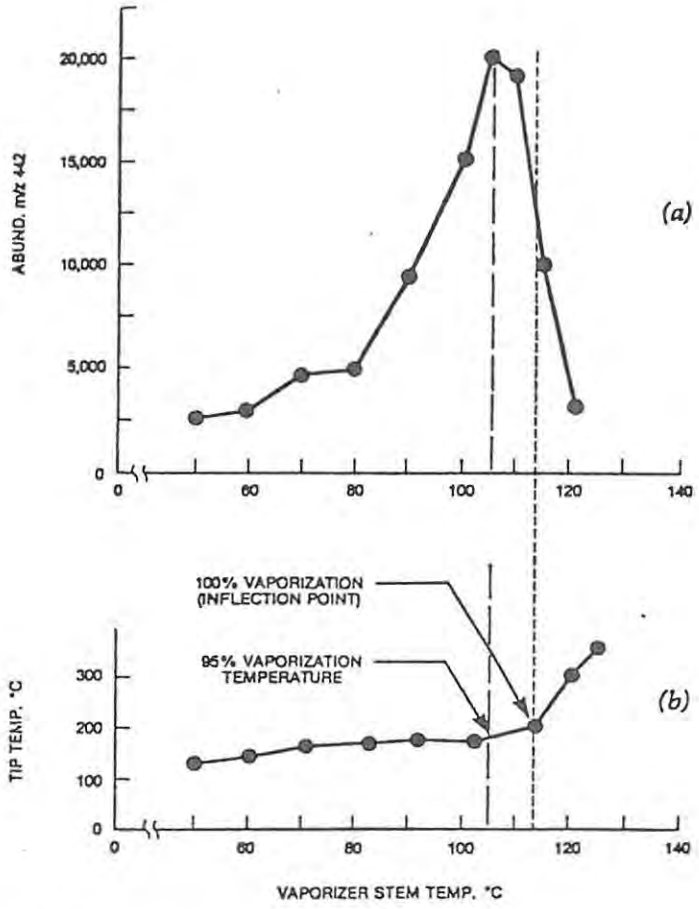
FIGURE 6.2 Fluid temperature in a TSP probe
Reprinted from H.P 5998A Thermospray User's Manual (144).



The optimum probe temperature for analysis varies according to the composition of the mobile phase (145, 150, 159). As the water content increases, the heat of vaporization increases and higher temperatures are required to achieve maximum ion formation. A new probe survey scan must therefore be conducted for each different LC solvent employed.

FIGURE 6.3 (a) Signal abundance of an arbitrary ion (*m/z* 422 $[M + H]^+$) *versus* vaporizer stem temperature; (b) vaporizer tip temperature *versus* stem temperature showing the inflection point.

Reprinted from Goodley P.C. (145).



6.2.2.2 Electron Filament

The use of an external source of high energy electrons to enhance TSP sensitivity has been previously mentioned. Electrons from the filament penetrate into the vapour chamber and assist in the formation of reagent ions from the buffered mobile phase. The abundance of analyte ions for the filament-on mode always exceeds that obtained for the filament-off mode and, therefore, the use of a filament is advocated in cases where maximum sensitivity is required. It is also recommended in cases where a high proportion of organic solvent is employed in the LC mobile phase (this effect is discussed in Section 6.2.2.3).

6.2.2.3 Solvent Composition

Thermospray operation requires the presence of a volatile buffer in the HPLC solvent (150). The mobile phase must, therefore, readily dissolve the buffer. Solvents such as acetonitrile in water and methanol in water comply with this requirement. Methanol, however, provides greater sensitivity in terms of ion abundance than acetonitrile because of the proton affinity differences between the two solvents (145).

It is well known that TSP sensitivity is enhanced as the proportion of buffer in the LC effluent is increased (145). In cases where it is mandatory to use a high percentage of organic solvent, the subsequent reduction in ion abundance (*i.e.* sensitivity) can be minimized by operating the TSP-MS in the filament-on mode.

The sensitivity of TSP analysis and the characteristics of a TSP spectrum are influenced by both the type of buffer and the concentration of buffer used in the mobile phase (150, 158). Although the information content of a spectrum does not appear to be significantly altered by the type of buffer utilized, an ammonium acetate buffer has been shown to provide the greatest sensitivity. It provides the best results when used at a 0.1M concentration.

6.3 STUDY OBJECTIVES

The purpose of this study was to determine the molecular masses of the two metabolites of mephenoxalone that were present in extracts of human urine samples using combined HPLC-MS under TSP ionizing conditions. The filament-on mode was employed for more efficient ionization. The use of a reverse-phase column enabled both the rapid analysis of the urine extracts and the resolution of mephenoxalone and the two metabolites. The TSP spectra that were generated permitted not only the determination of molecular masses but also enabled a distinction to be made between conjugated and unconjugated metabolites.

6.4 THERMOSPRAY ANALYSIS OF MEPHENOXALONE AND ITS METABOLITES

The human biotransformation products of mephenoxalone have been identified by Morrison (6) and by Eckhardt *et al.* (28) and are depicted in Fig.1.2. Morrison isolated four transformation products, Metabolites IIIa, IIIb and their glucuronic acid conjugates, using a countercurrent distribution procedure. The identities of the free phenolic compounds were established by paper chromatographic techniques, while the conjugates were subjected to enzymatic cleavage and acid hydrolysis prior to chromatographic analysis. Eckhardt *et al.* (28) isolated numerous metabolites by a series of acidic, basic and neutral chloroformic extractions of urine. Of the recovered compounds, Metabolites I and II were identified by thin layer chromatography (TLC). Five other metabolites were ascertained to be conjugated derivatives; their free form structures (Metabolites III, IV, V, VI and VII) were identified after acid and enzyme hydrolysis of urine followed by extraction and TLC analysis.

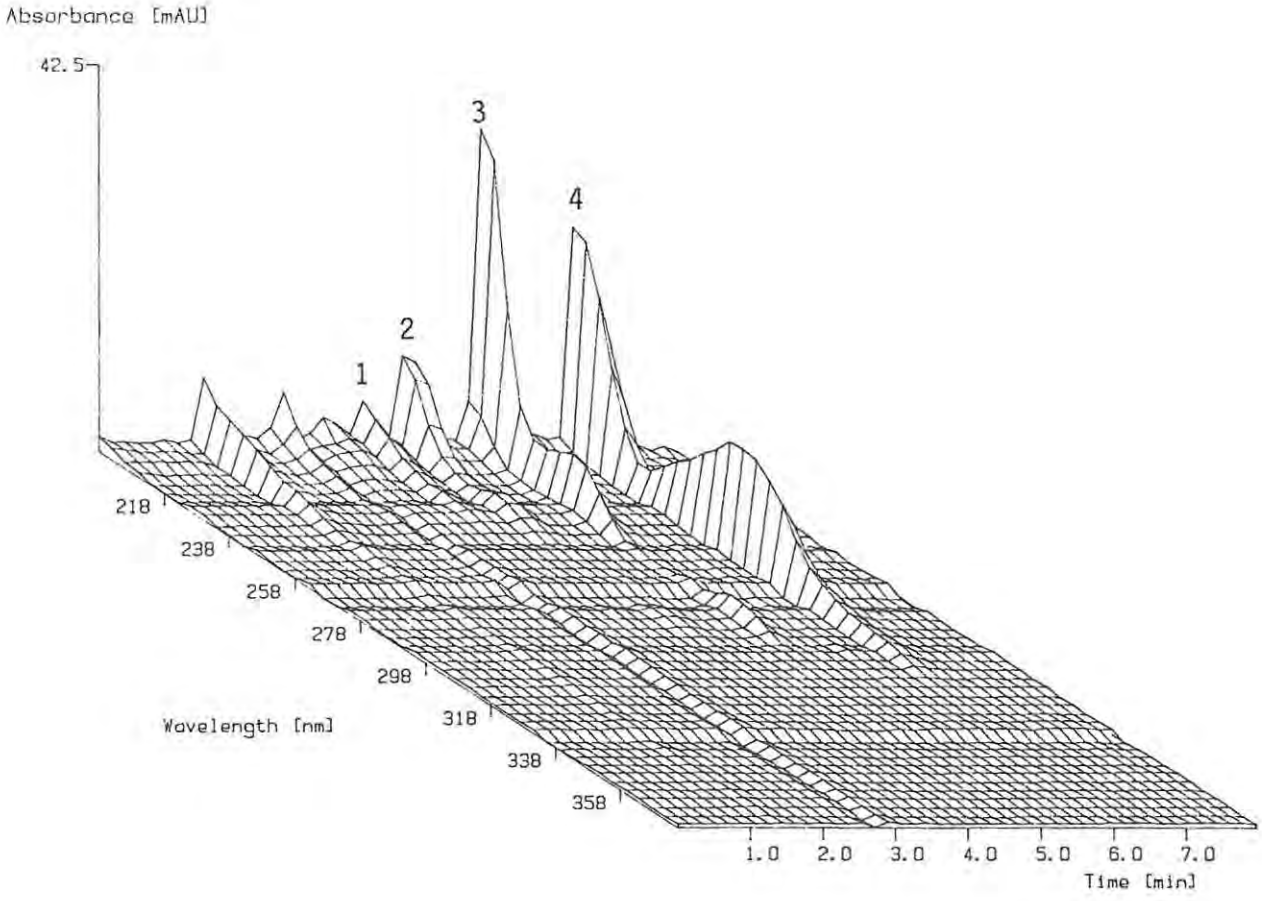
Urine samples that were collected from human subjects after oral administration of mephenoxalone and subsequently analyzed according to Section 3.6.1.3 revealed the presence of two UV-absorbing metabolites. Their ultraviolet spectra (Fig.6.4) were determined using an HP 1040A photodiode array detector placed in-line with the HPLC system. The spectra of the metabolites show absorption maxima similar to those of the parent drug. From these spectra, however, it was not possible to ascertain whether or not these metabolites were conjugates because the conjugating moiety (glucuronic acid) has no effect on the characteristics of the absorption spectrum (160). It was, however, possible to determine the molecular mass of each compound using thermospray analysis, thereby enabling a distinction to be made between conjugated and non-conjugated metabolites.

The use of the thermospray technique for the analysis of several drugs (and their metabolites) in biological fluids has been reported (155). Data were acquired in the scan mode and the molecular masses of the compounds were thus determined. The TSP spectra generated were simple and few fragment ions were observed.

FIGURE 6.4 Three-dimensional "spectrochromatogram" (absorbance *versus* wavelength *versus* time) showing a urine extract (Section 6.4.1.2) containing Metabolites IIIa[1] and IIIb[2], mephenoxalone[3] and phenacetin[4]

Chromatographic conditions	:	as in Section 2.4.2
Injection volume	:	10ul
Bandwidth at sample wavelength (200nm)	:	8nm
Bandwidth at reference wavelength (320nm)	:	20nm
Scan rate	:	1 sec^{-1}

Viewed at an angle of 35°, from the left.



Data acquisition modes offered by the TSP system utilized in this study comprise both a scan mode and a selected ion monitoring (SIM) mode. For quantitative purposes, SIM is preferable because of the enhanced sensitivity and selectivity that this acquisition process affords (126). Qualitative analysis, however, is routinely accomplished by repetitive mass scanning over a range of molecular masses. A total ion count (TIC) chromatogram is then generated by summation of all the ions in each mass spectrum. The identification of a compound is achieved by examining the mass spectrum at the time corresponding to the top region of the chromatographic peak. To confirm the presence of the ions observed in this spectrum, a reconstructed mass (ion) chromatogram can be generated, after the LC run, by means of computer reconstruction methods.

In this study, positive ion spectra were obtained using the filament-on mode, and mephenoxalone and its metabolites were separated by isocratic elution using a buffered mobile phase. TIC chromatograms were generated by scanning a suitable mass range and the spectrum of each compound was subsequently obtained using computer reconstruction methods. Prior to the analysis, verification of TSP operating conditions and optimization of parameters was necessary.

6.4.1 Materials and Apparatus

6.4.1.1 Materials

Mephenoxalone (experimental powder batch) was supplied by Adcock-Ingram Laboratories Ltd., Johannesburg, South Africa.

Analytical grade ammonium acetate (Saarchem (Pty) Ltd, Muldersdrift, South Africa) was obtained commercially.

Methanol and acetonitrile (Burdick and Jackson Laboratories, Muskegon, Mich., USA) were distilled-in-glass grade. HPLC-grade water was prepared as in Section 2.3.1.

TSP ionization reagent (part number 8500-4410) and polypropylene glycol (part number 8500-4409) were supplied by Hewlett-Packard Co. Palo Alto, Cal., USA.

6.4.1.2 Source of Biological Samples

Urine collected six hours after oral administration of 400mg mephenoxalone dispersed in 150ml water to a human volunteer (as in Section 5.1.1) was stored at -20°C until required. It was thawed at room temperature prior to use.

6.4.1.3 Apparatus

The TSP HPLC-MS system used throughout this study is depicted in Fig.6.5. Data acquisition and processing was performed using the Chemstation data system provided by Hewlett-Packard.

6.4.2 Experimental

6.4.2.1 Sample Preparation

Mephenoxalone stock solution (1mg/ml) was prepared as in Section 3.4.1.1. This solution was diluted 1 in 50 with water to produce a 0.02mg/ml solution.

Ionization solution (0.1M ammonium acetate) was prepared by mixing 490ml of water with 2 ampoules of TSP ionization reagent.

Human urine samples (see Section 6.4.1.2) were extracted as in Section 3.6.1.3.

6.4.2.2 TSP HPLC-MS Conditions

TSP HPLC-MS system : As in Section 6.4.1.3.

Mass Spectrometer Operating Conditions:

Mass filter	:	quadrupole type
Ionization mode	:	filament-on
Ionization energy	:	2100 eV
Ion source temperature	:	270°C

Analyzer manifold pressure	:	2×10^{-6} Torr
Low vacuum pump pressures	:	TC1 = 0.1 Torr
		TC2 = 0.7 Torr
Data acquisition mode	:	scan

Mass calibration and tuning were achieved through the data system using the TSP Tune file called TXTUN1.U., a pre-programmed instruction on the Chemstation data system.

HPLC Operating Conditions:

Temperature	:	ambient
Mobile phase flow rate	:	1.0ml/min

The mobile phases used for this study were as follows:

Eluent A.	Probe survey solution
Eluent B.	Standard tuning solution
Eluent C.	Acetonitrile - 0.1M ammonium acetate (35/65)

Mobile Phase Preparation

Eluent A was prepared by mixing 375ml ionization solution (Section 6.4.2.1) with 125ml methanol.
Eluent B was prepared by mixing 375ml HPLC-grade water, 115ml methanol, 2 ampoules of ionization reagent and 1 ampoule of polypropylene glycol.
Eluent C was prepared by mixing 175ml acetonitrile with 325ml 0.1M ammonium acetate buffer.

No corrections were made for volume changes which occurred upon mixing. The solvent mixtures were degassed and filtered through a 0.45µm filter (Type HVLP, Millipore Corp., Bedford, Mass., USA) prior to use.

FIGURE 6.5 TSP HPLC-MS system

(1) Model 112 solvent delivery system (Beckman Instruments, Inc., San Ramon, Cal., USA)

(2) Model 3XL soft seal injector equipped with a 10ul loop (Scientific Systems, Inc., Philadelphia, Pa., USA)

(3) C18 reverse-phase 5um column (as in Section 2.5.2.2)

(4) Model 5988A mass spectrometer (Hewlett-Packard Co,, Palo Alto, Cal., USA) interfaced to the HPLC system

(5) Standard Hewlett-Packard thermospray interface (Option 015)

(6) Standard computer hardware (Hewlett-Packard Co., Palo Alto, Cal., USA).

6.4.2.3 Thermospray Verification

After setting up the LC-MS system according to the manufacturer's instructions, it was necessary to verify that the system was operating correctly. An isocratic probe survey using Eluent A was performed. The 95 percent vaporization temperature was calculated and recorded. The thermospray system was then tuned in the positive ion, filament-on, extended mass range mode using Eluent B and the optimum control temperature. The tune report was automatically generated.

6.4.2.4 TSP Analysis of Mephenoxalone and its Metabolites

Using Eluent C a probe survey was conducted, starting at a stem temperature of 100°C and ending at 130°C, at a rate of 3°C increase per minute. The 95 percent vaporization temperature was then calculated and this value was employed as the stem temperature setpoint for all further TSP analyses.

To determine the sensitivity of the TSP technique for the analysis of mephenoxalone, 10µl aliquots containing 10µg mephenoxalone (in water) were injected onto the column. Data were initially acquired by scanning across a mass range of 3 to 350amu. A sample containing 200ng of mephenoxalone was then injected. The mass range was then reduced so as to scan from 200 to 250 amu and the samples were re-injected.

Urine samples, extracted according to Section 3.6.1.3, were analyzed by injecting 10µl aliquots of the extracts into the HPLC-MS system. Data were acquired by scanning the following mass ranges:

3 to 275amu
150 to 220amu
220 to 265amu

6.4.3 Results and Discussion

6.4.3.1 Verification of Correct TSP Operation

No interpretation is required of the TSP tune results (144). If a report is generated, the system has passed the TSP tune and is ready to run samples. However, it is useful to compare the report to previous tune reports so that developing problems can be ascertained. Several specifications for the results of the report have been laid down; the three ions (*m/z* 268.1, 442.6 and 558.6) should have relative abundances of >20%, 100% and >20%, respectively, with an abundance for *m/z* 442.3 of over 6000. In addition the peaks should be evenly spaced in the spectrum. The tune report generated using a probe stem setpoint of 120°C (obtained from the probe survey scan) is depicted in Table A6.1. It shows evenly spaced peaks and complies with all the specifications except for that concerning the abundance of the *m/z* 442.6 ion. However, this had no serious implications.

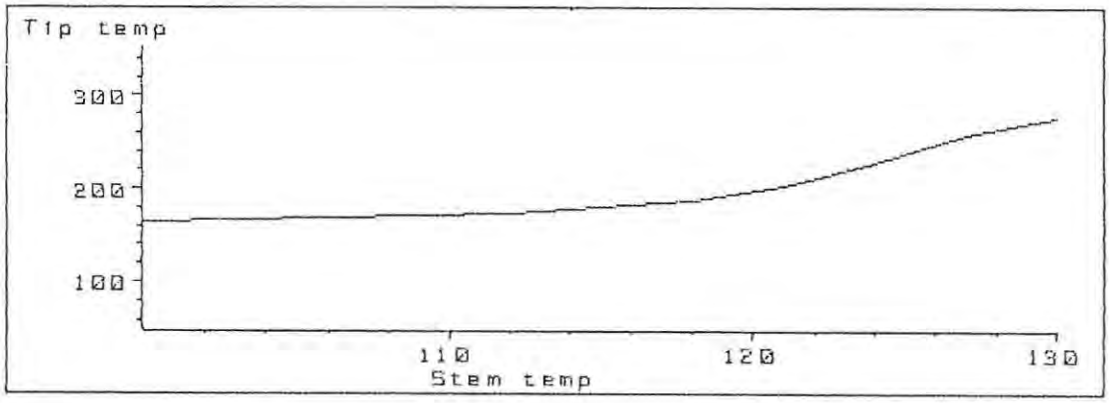
6.4.3.2 Choice of Mobile Phase

An ammonium acetate buffer (0.1M) was adopted for this study for the reasons previously discussed (Section 6.2.2.3). A preliminary investigation using HPLC with UV detection was conducted to determine how the elution profile of mephenoxalone and its metabolites would be affected by the addition of this buffer to the mobile phase. Ultraviolet detection at 220nm was employed because this represents a wavelength of maximum absorbance for mephenoxalone and the two metabolites without interference from the buffer. The mobile phase used was acetonitrile-0.1M ammonium acetate buffer (35/65). The chromatogram obtained from a urine extract using this phase was compared to a chromatogram obtained from the same extract using a mobile phase consisting of acetonitrile-water in the same proportions. It was found that the retention times were unaltered by the presence of buffer and this mobile phase was therefore adopted for TSP analysis of mephenoxalone.

6.4.3.3 Probe Survey Results

Figure 6.6 shows the probe survey plot obtained using an acetonitrile-0.1M ammonium acetate (35/65) mobile phase. The inflection point (100% vaporization temperature) occurred at 119°C and the optimum stem setpoint (95% vaporization temperature) was therefore 112°C.

FIGURE 6.6 Probe survey plot using an acetonitrile-0.1M ammonium acetate (35/65) mobile phase

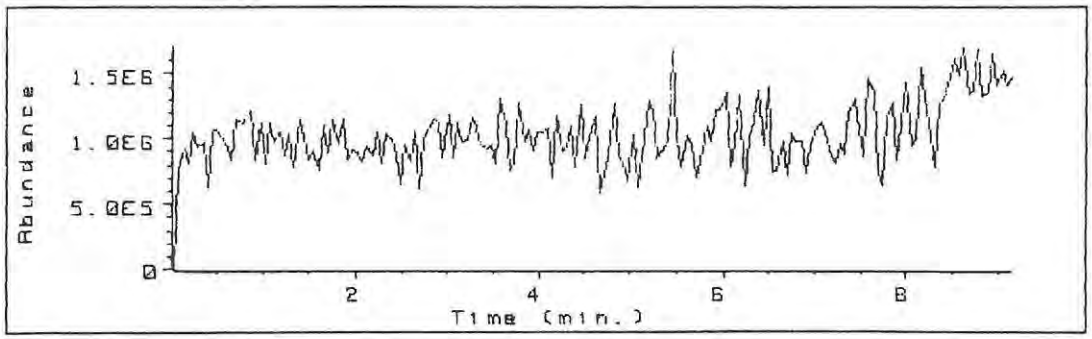


6.4.3.4 TSP Spectrum of Aqueous Mephenoxalone

The TIC trace obtained by full mass scanning from 3 to 350amu of a 10µl injection containing 10µg mephenoxalone in water was devoid of any readily identifiable peak (Fig.6.7). A high solvent ion abundance was evident and the baseline fluctuations were large. The poor quality of the acquired chromatogram was attributed to several factors. The regular oscillations in background intensity were the result of flow fluctuations introduced by the HPLC pump. This effect has been previously reported in the literature and should be avoided as far as possible by using a constant-flow pumping mechanism. It is known that the mass scan range must often be limited to a small range

in order to increase the scanning rate and thereby enhance the sensitivity of the analysis (146, 155). Useful mass spectra below *m/z* 150 are often difficult to obtain even with the aid of computer background subtraction methods. The detection limit of an analysis is therefore enhanced by both reducing the range of masses scanned (with scanning above *m/z* 150 wherever possible) and using an LC delivery system which provides minimal flow fluctuations.

FIGURE 6.7 TIC trace obtained by full mass scanning from 3 to 350amu of a 10µl injection containing 10µg mephenoxalone in water.



Despite the lack of a mephenoxalone peak in the TIC (Fig.6.7), a mass spectrum of the compound was generated with the aid of computer reconstruction methods; a specific mass ion chromatogram of an expected ion *m/z* 241 (the molecular mass of ammoniated mephenoxalone) was generated (Fig.6.8) and, using the retention time of this ion, a mass spectrum was obtained from the corresponding time on the TIC. However, the high abundance of solvent ions necessitated the use of a computer background subtraction technique to generate the final mass spectrum of mephenoxalone (Fig.6.9). The primary ions observed are the $[M + H]^+$ ion and the $[M + NH_4]^+$ ion, with masses of 224 and 241amu, respectively. The base peak comprised the ammoniated ion.

FIGURE 6.8 Reconstructed ion range (*m/z* 241.0-241.9) chromatogram

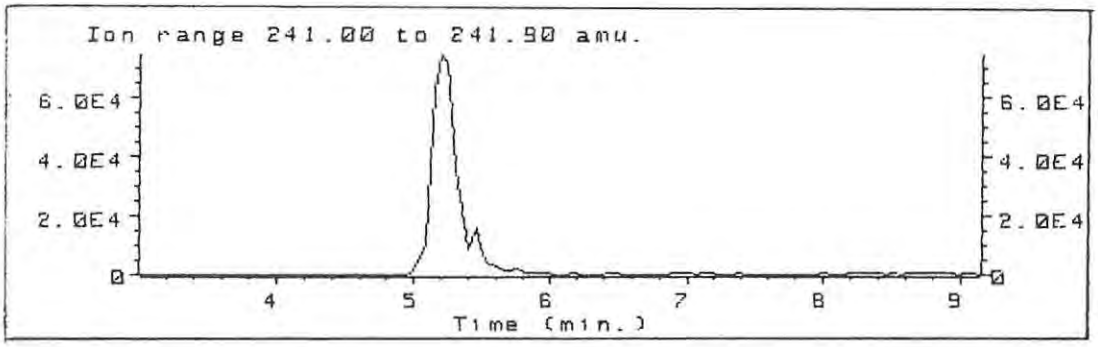
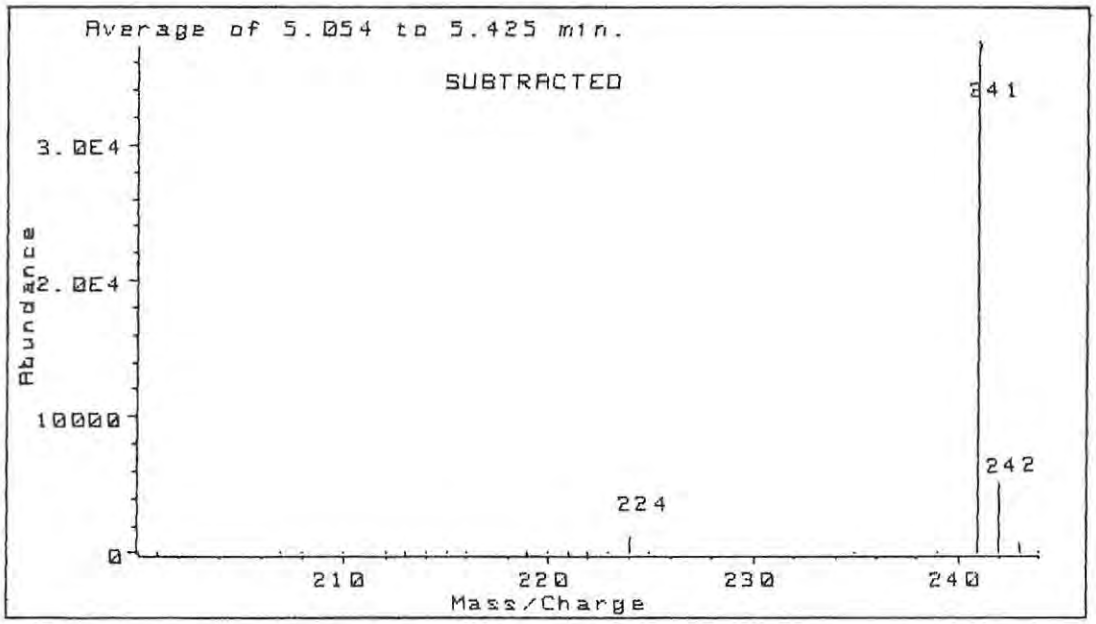


FIGURE 6.9 Subtracted mass spectrum of mephenoxalone



The above procedure was laborious and an alternative approach for generating spectra, using a smaller scanning range, was investigated. A markedly improved TIC trace was thereby obtained. By re-injecting the 10µg aqueous mephenoxalone sample and scanning from 200 to 250 amu, a distinct peak was observed in the TIC trace (Fig.6.10). The mass spectrum, taken at the top of the chromatographic peak, is shown in Fig.6.11. The ammoniated and protonated molecular adduct ions are formed, as are ions corresponding to *m/z* 242 and 243.

FIGURE 6.10 TIC trace obtained by mass scanning from 200 to 250amu of a 10µl injection containing 10µg mephenoxalone in water

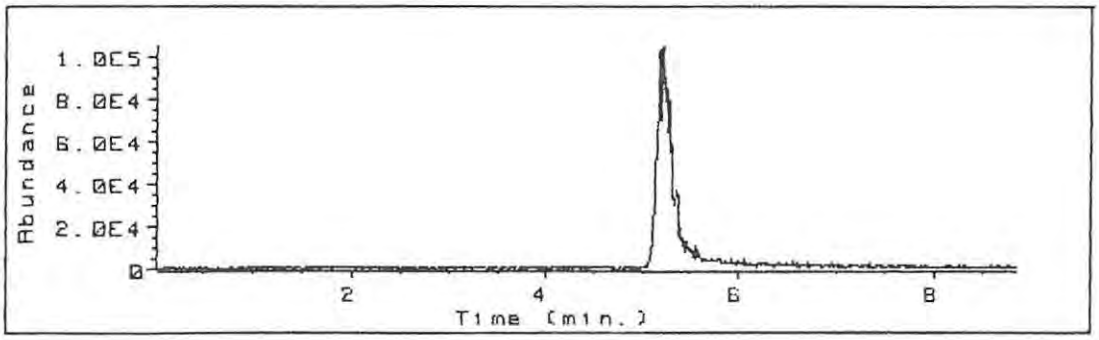
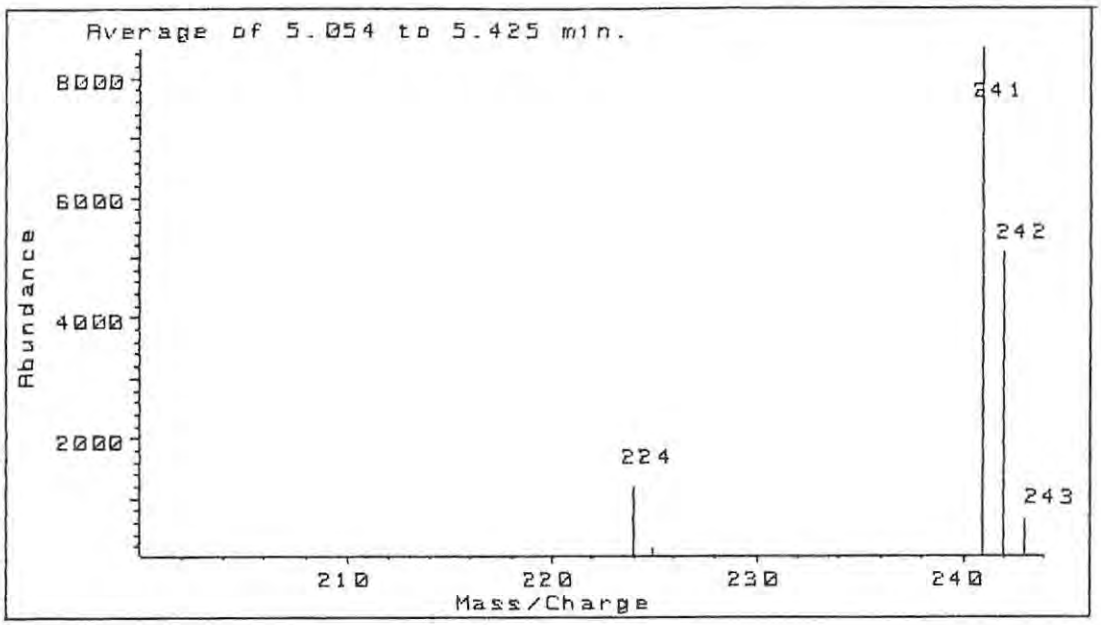


FIGURE 6.11 Mass spectrum of mephenoxalone



A 10µl aqueous injection containing 200ng mephenoxalone was injected to determine the feasibility of using TSP for analyzing the low levels of the drug found in biological fluids. The TIC trace (Fig.6.12) shows that mephenoxalone can be detected at this level despite the high background noise. Figure 6.13 depicts the mass spectrum, which had to be acquired by computer background subtraction because of the effect of pump-induced flow fluctuations. The oscillations in background noise made it difficult to distinguish between solvent ions and sample ions in a spectrum taken directly from the TIC

chromatogram, and a subtracted spectrum was therefore obtained by subtracting an average spectrum of background ions from an average spectrum of the chromatographic peak, both spectra being taken over the peak of interest. The subtracted spectrum clearly shows the *m/z* 241 and 242 ions. An *m/z* 222 ($[M - H]^+$) ion was also observed but no protonated sample molecule (*m/z* 224)was seen. It appears that a concentration-dependent phenomenon exists, where protonation does not occur at low concentrations of mephenoxalone.

FIGURE 6.12 TIC trace obtained by mass scanning from 200 to 250amu of a 10µl injection containing 200ng mephenoxalone in water

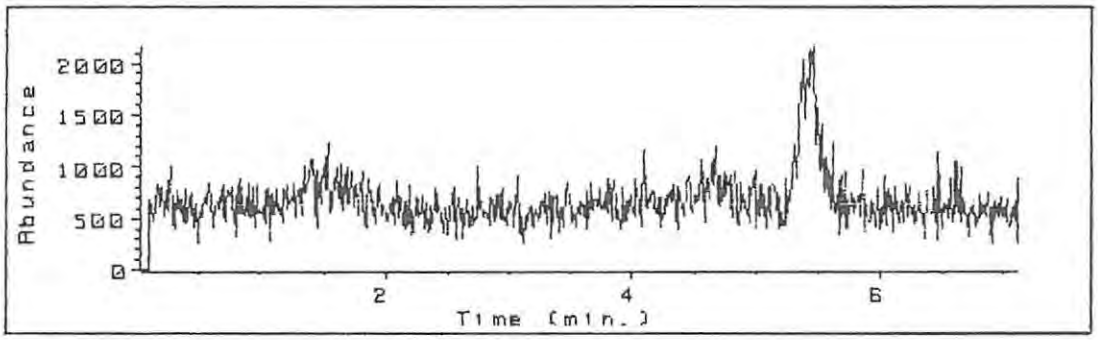
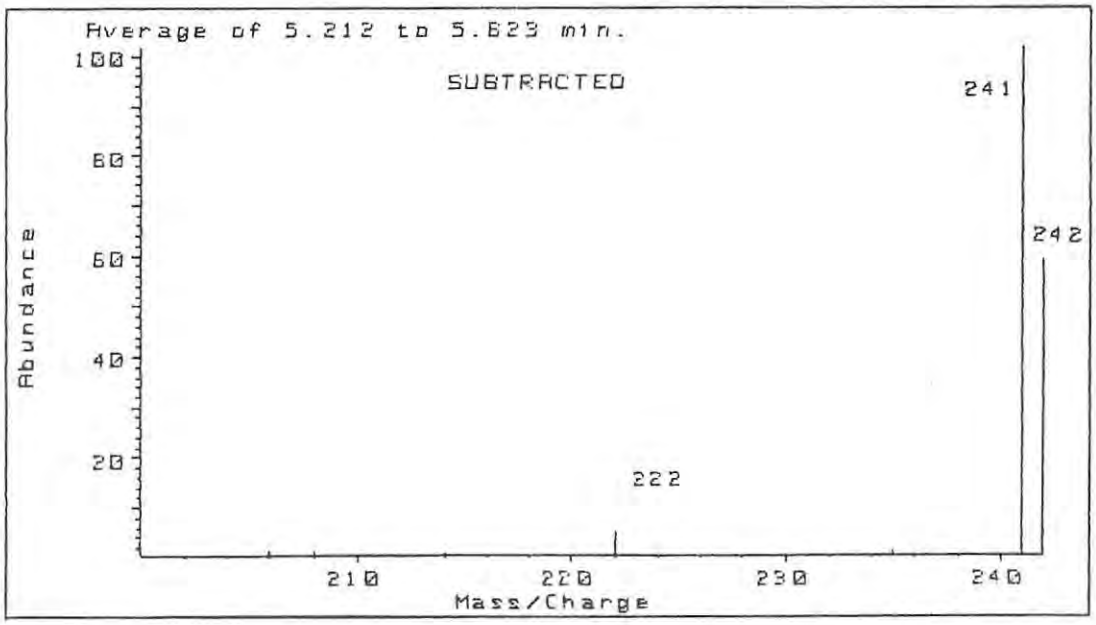


FIGURE 6.13 Subtracted mass spectrum of mephenoxalone



6.4.3.5 TSP Spectra of Mephenoxalone and its Metabolites in Human Urine

A model LC-UV detector (Pye Unicam Ltd., Cambridge, England), was employed in line with the TSP system. Figure 6.14 shows the UV chromatogram for the separation of the parent drug from its metabolites after a 10µl injection of a human urine extract, while the TSP HPLC-MS TIC trace (obtained at the same time using a scan range from *m/z* 220 to 260) is shown in Fig.6.15. Both the UV and the TSP traces revealed the presence of 3 sample components, with retention times of 3.4, 4.3 and 5.3 minutes. The first two compounds to be eluted were later identified as Metabolite IIIa and Metabolite IIIb, respectively.

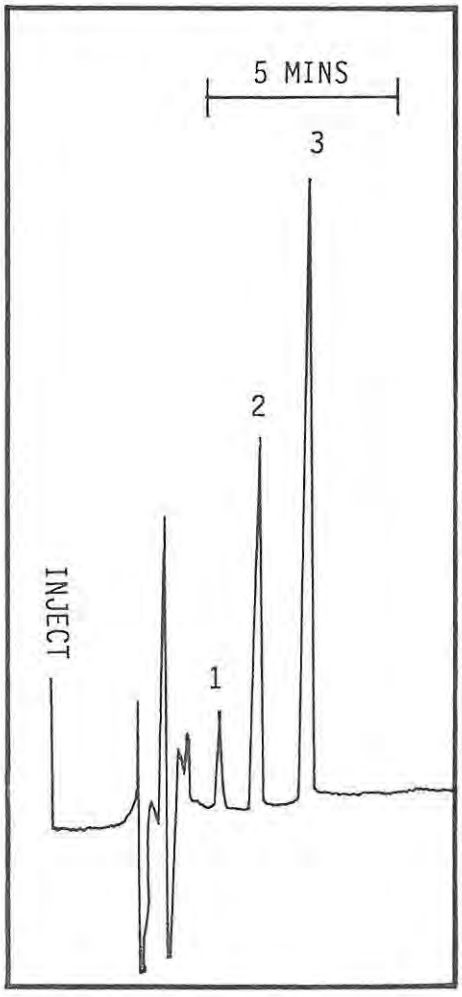
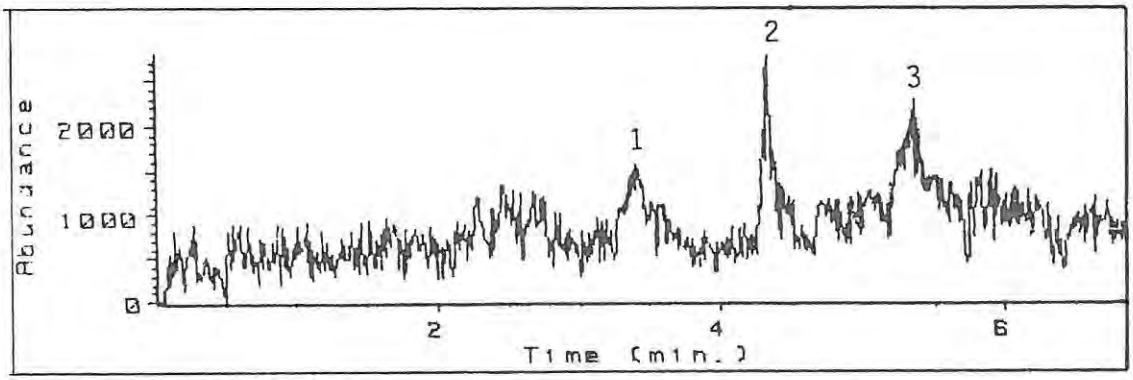


FIGURE 6.14 UV chromatogram showing a urine extract (section 6.4.1.2) containing Metabolites IIIa[1] and IIIb[2], and mephenoxalone[3] (chromatographic conditions as in Section 2.4.2; 10µl injection volume)

The subtracted average mass spectrum of Metabolite IIIa (Fig.6.16(a)) reveals the presence of ions of mass 242, 257 and 258amu. The 242amu ions are not significantly abundant. The only possible metabolite that these ions can be derived from is Metabolite IIIa in its unconjugated form. If the metabolite was a conjugated derivative which had been fragmented by TSP to produce the above ions, then glucuronic acid fragments would also have been present. However, data acquired from a mass scan range of 150 to 200amu showed that no ions of mass 176 or 194amu were present (these ions would represent the glucuronide fragment and its ammoniated derivative, respectively).

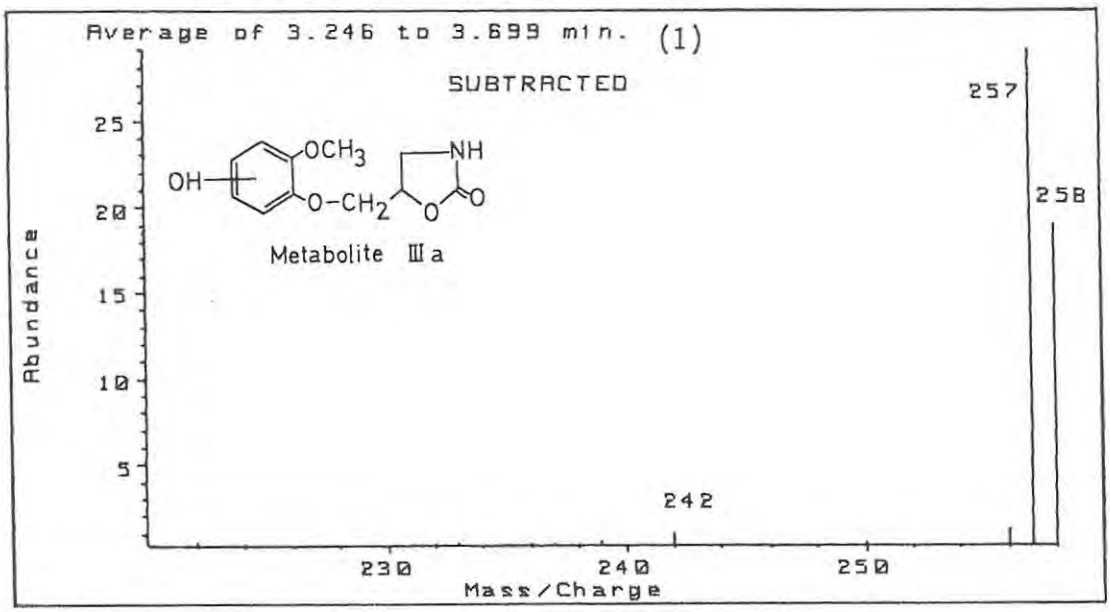
FIGURE 6.15 TIC trace obtained by mass scanning from 220 to 260amu of 10ul inject of a urine extract containing Metabolite IIIa, Metabolite IIIb[2], and mephenoxalone 3.

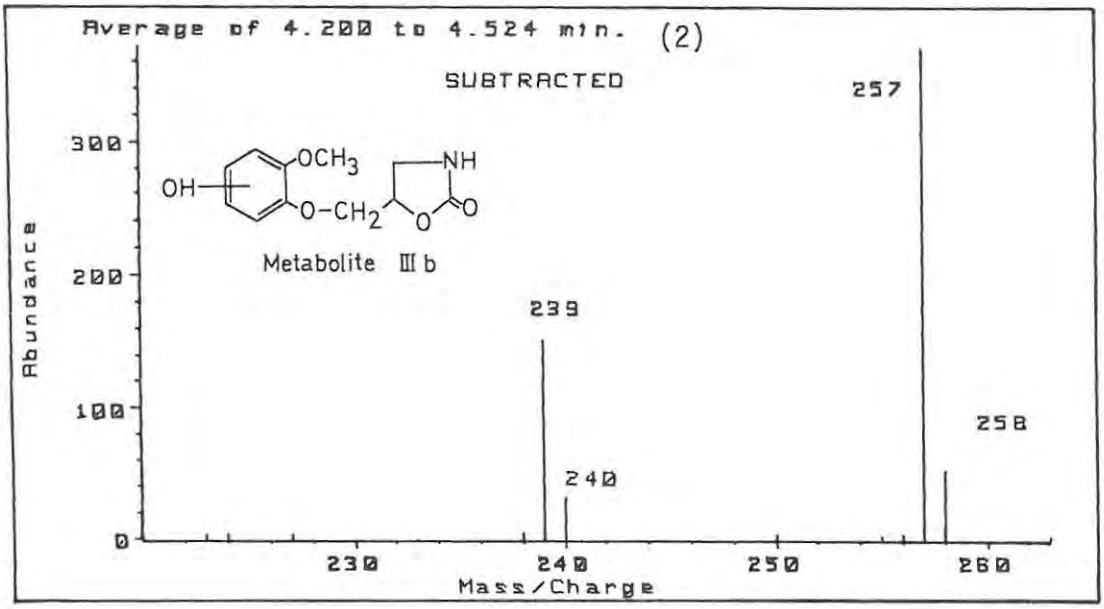


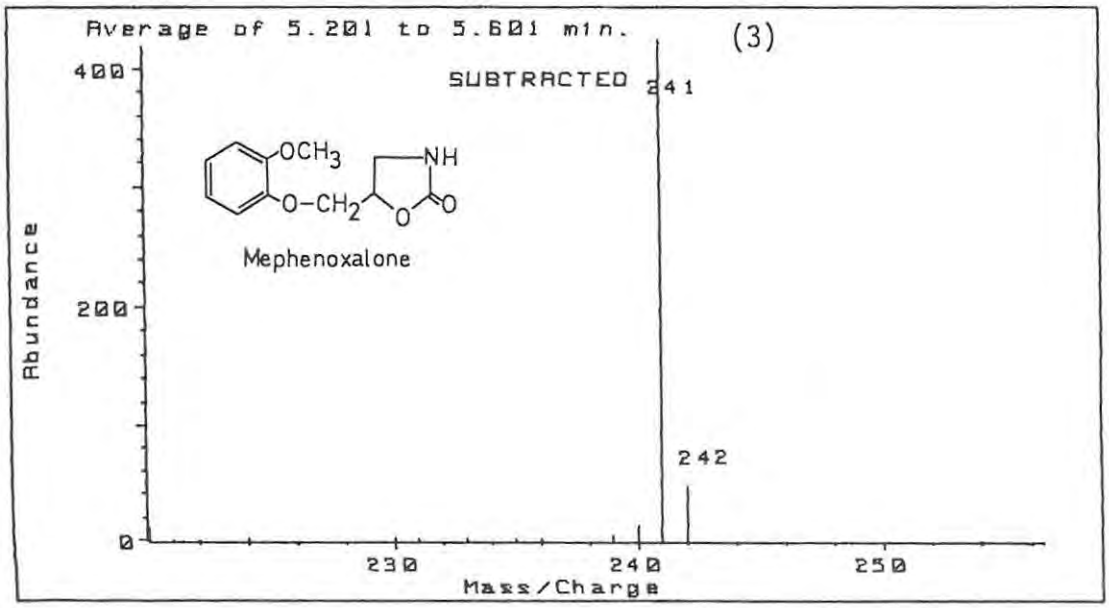
The average mass spectrum of Metabolite IIIb is shown in Fig.6.16(b). Ions with masses of 239, 240, 257 and 258amu, corresponding to $[M]^+$, $[M + 1]^+$, $[M + 18]^+$ and $[M + 19]^+$, respectively, are formed. Metabolite IIIb was confirmed as being the free phenolic derivative as described earlier.

The mass spectrum of mephenoxalone isolated from urine (Fig.6.16(c)) shows the same ionization products as those formed after TSP analysis of 200ng of the drug in water. Both Metabolites IIIa and IIIb produce spectral base peaks at m/z 257. These ions correspond to the ammoniated derivative of the unconjugated phenolic metabolites. However, there is a difference between the spectra of the two

FIGURE 6.16 Subtracted mass spectra of Metabolites IIIa(1) and IIIb(2), and mephenoxalone (3)







6.5 CONCLUSIONS

The molecular masses of the two mephenoxalone metabolites that were extracted from urine were examined by TSP HPLC-MS. A mobile phase of acetonitrile - 0.1M ammonium acetate buffer provided adequate resolution between the 3 peaks of interest, and chromatographic integrity was unaffected by the TSP interface.

The metabolites were ascertained to be unconjugated phenols, each of molecular mass 239amu. These results are in good agreement with the metabolite data published by Morrison (6). The thermospray spectra of mephenoxalone and its biotransformation products were relatively simple, consisting primarily of an $[M + NH_4]^+$ ion peak. The lack of fragment ions made structural elucidation impossible, thereby preventing the assignment of ring positions to the hydroxyl groups of the metabolites. Since the ammoniated ions were the base peaks, the analyte molecular ions would appear to react more favourably with ammonia than with water (140, 152).

In this study, two metabolites were extracted by liquid-liquid extraction of human urine samples. It has been reported that conjugates are not amenable to solvent extraction (56) and this confirms earlier findings that the metabolites present in the urine extracts were unconjugated. The difference in molecular mass between the metabolites and mephenoxalone was 16amu, which was ascertained to be the result of hydroxylation of the parent compound. The phenolic isomers thereby produced would be weakly acidic and, therefore, would not be extracted into an organic phase under alkaline conditions as was reported by Morrison (6).

As a detector for the HPLC analysis of drugs, the mass spectrometer has enormous potential. TSP is well suited to the analysis of biological fluids provided that the system is operated under optimum conditions. The spectra generated in this study were of the required quality for the molecular mass determinations but could have been improved by utilizing a solvent delivery system having better flow control.

metabolites in that IIIb forms the $[M]^+$ and $[M + H]^+$ ions, whereas IIIa does not. This may be the result of the greater relative abundance of the former compared to the latter. This concentration-dependent ionization effect has already been discussed. A summary of the ions formed upon TSP HPLC-MS of mephenoxalone and its metabolites is presented in Table 6.2.

TABLE 6.2. Summary of results obtained by TSP analysis of mephenoxalone and its metabolites.

	COLUMN LOAD PER INJECTION VOLUME	TIC PEAKS	COMPONENTS (RETENTION TIME IN MINUTES)	MOLECULAR MASS	SEPCTRAL *m/z* IONS	PROPOSED STRUCTURE
Aqueous mephenoxalone	10µg/10µl	1	Mephenoxalone (5.3)	233	224	$[M + H]^+$
					241*	$[M + NH_4]^+$
					242	$[M + NH_4 + 1]^+$
					243	$[M + NH_4 + 2]^+$
Aqueous mephenoxalone	200ng/10µl	1	Mephenoxalone (5.3)	223	241*	$[M + NH_4]^+$
					242	$[M + NH_4 + 1]^+$
Urine extract[a]	10µl[b]	3	1. Metabolite IIIa[c] (3.4)	239	257*	$[M + NH_4]^+$
					258	$[M + NH_4 + 1]^+$
			2. Metabolite IIIb[c] (4.3)	239	239	$[M]^+$
					240	$[M + H]^+$
					257*	$[M + NH_4]^+$
					258	$[M + NH_4 + 1]^+$
			3. Mephenoxalone (5.3)	233	241*	$[M + NH_4]^+$
					242	$[M + NH_4 + 1]^+$

* Most abundant ion (Base peak)
a Urine collected from subject DW after oral administration of mephenoxalone (Section 5.1.1.7)
b Exact column load unknown because no pure metabolites were available for quantification
c Proposed structures of Metabolites are shown in Fig.1.2

CHAPTER SEVEN

CONCLUSION

There is a severe lack of pharmacokinetic studies on mephenoxalone reported in the literature, possibly due to the lack of a rapid, sensitive, precise analytical method for the determination of the drug in biological fluids. The method which was developed during the course of this study was found to be readily applicable to pharmacokinetic studies in humans. The drug was extracted from serum and urine using simple liquid-liquid extraction procedures, and the concentrations were subsequently determined by reverse-phase HPLC using UV detection at 200nm. The detection limits for mephenoxalone in serum and urine were 0.04 and 0.2µg/ml, respectively.

Mephenoxalone is usually administered in oral doses of 400mg three times a day. However, prior to multiple dose pharmacokinetic studies, it is important to characterize single dose kinetics. Since it was not possible to perform intravenous studies, an oral dose study was conducted instead. Solution data were not available because of the poor solubility of mephenoxalone in water and the test dose was, therefore, administered as a dispersion of the pure powder in 150ml water.

The concentrations of mephenoxalone which could be expected in serum and in urine following a single oral dose of the dispersion were established in the pilot study; this study also yielded information on the sampling frequency required to characterize the absorption and disposition curve.

A single dose dispersion study was then conducted using six young, healthy volunteers. Five of these subjects participated in a subsequent clinical study to assess the bioavailability of mephenoxalone from a newly developed solid oral dosage form containing a combination of mephenoxalone, paracetamol and codeine phosphate. The most meaningful results were obtained from the statistical moment parameters MRT, MAT and MDT, and from the model-independent parameters C_{max}, t_{max}, λ_z, $t_{1/2}$ and AUC_{∞}. The trial data were also fitted to

one-body and two-body compartment models using the computer program NONLIN but neither of these models, which incorporated only one absorption rate constant, were able to account for the rapid absorption phase; the predicted peak concentrations were always lower and later than the experimentally observed values. The incorporation of a discontinuous absorption phase to more accurately characterize the absorption process was not investigated.

The absorption of mephenoxalone from the dispersion was rapid, with the peak concentration of approximately 8μg/ml appearing after about 1 hour. Absorption of the compound from the tablet dosage form was slightly faster, with the peak concentration being higher (12μg/ml) and earlier (0.6 hours) in all but one of the subjects. The tablet had a shorter *in vivo* dissolution time and consequently was absorbed more rapidly than the dispersed dosage form. The *in vitro* dissolution studies showed that mephenoxalone dissolved more rapidly from the tablet than from the dispersion, thus supporting the *in vivo* findings. It was subsequently revealed by the manufacturer that various excipients had been included in the formulation to promote the release and dissolution of mephenoxalone from the tablet. However, the faster *in vivo* dissolution of the tablet is not expected to be clinically significant since mephenoxalone is a low potency agent with a moderate dose-response curve (16,24).

In this study, the compound was extensively metabolized with only about 1 percent of the administered dose being excreted as unchanged mephenoxalone in urine after 24 hours. Two of the metabolites present in extracted urine samples were subjected to thermospray HPLC-MS analysis and were ascertained to be the unconjugated hydroxylated derivatives of mephenoxalone. However, elucidation of the precise structures of these metabolites was not possible because of the lack of molecular fragmentation associated with the thermospray technique.

From the clinical trial data, the post-absorptive phase was generally characterized by a fairly rapid mono-exponential decrease in the serum concentration of mephenoxalone. The elimination half-life for the compound was approximately 3 hours.

There were, however, considerable individual variabilities, both intra-subject and inter-subject, in the trial data. While subjects WA and DW consistently displayed rapid rates of absorption and elimination in both trials, subject KR displayed a consistently slow rate of elimination. The long elimination half-life for mephenoxalone in KR may be explained either by inter-subject variation or because she had a mild case of Gilberts' Syndrome. Intra-subject variations may be responsible for the results obtained from subjects KZ and MG. Thus, although KZ absorbed mephenoxalone rapidly from both test doses, she had a much slower rate of elimination after ingestion of the tablet dosage form. This discrepancy can also be explained by a reduction in the metabolic rate as a result of dieting during the 4 month separation period between trials. Subject MG had a consistently rapid elimination half-life for both trials but absorbed mephenoxalone more slowly from the tablet dosage form than from the dispersion; no explanation other than individual variation or the effect of codeine on gastro-intestinal mobility can be offered for these results.

It is obvious that the difficulties of interpreting the data obtained in these studies lie not only with the inadequate characterization of the absorption process but also with the variations amongst the individuals. In addition, the sample sizes used for the clinical trials were not large enough to draw valid conclusions regarding the significance of any differences between the results of males and those of females.

The inclusion of paracetamol and codeine phosphate into the tablet dosage form did not appear to alter the pharmacokinetic characteristics of mephenoxalone. Furthermore, these compounds did not interfere with the analytical procedure because they were not extracted using the liquid-liquid extraction technique employed for isolating mephenoxalone from biological fluids.

A major drawback of the clinical studies was the lack of i.v. data which would have enabled the calculation of absolute bioavailability, volume of distribution and clearance. The parameter values reported by Morrison (6) for beagle dogs indicate that mephenoxalone has a volume of distribution of 110 to 120 percent of body mass, and that it

is almost completely absorbed after oral administration. It was also reported that mephenoxalone exhibits non-linear elimination kinetics upon repeated administration of high doses.

In this study, there was no evidence of non-linear elimination kinetics. However, only single oral dose pharmacokinetics were investigated and the behaviour of the drug in humans after multiple dosing is not known. The possibility of non-linearity does exist because the conjugation process, *i.e.* the primary elimination route of mephènoxalone, may become saturated, especially in hepatically compromised patients.

No protein binding data for mephenoxalone appear in the literature and an investigation was therefore conducted using the equilibrium dialysis method. The binding of the drug to human serum proteins appeared to be variable but no conclusive results were obtained.

The work remaining to be conducted on mephenoxalone seems endless but, as a beginning, this thesis will hopefully provide a background as well as some insight into the pharmacokinetic behaviour of this drug in humans.

APPENDIX

TABLE A3.1 Statistical intepretation of storage stability data.

The measured percentage response difference (D) between stored and freshly prepared samples was calculated using the following equation:

$$D = \frac{GM_y - GM_x}{GM_x} \cdot 100 \quad (\%)$$

Where GM_x is the geometric mean of $x_1, x_2 \ldots x_m$
GM_y is the geometric mean of $y_1, y_2 \ldots y_n$

GM_x and GM_y were calculated as follows:

$u_i = \ln X_i \; (i = 1,2..m)$

$v_i = \ln Y_i \; (i = 1,2..n)$

$$\bar{u} = \sum_{i=1}^{m} \frac{u_i}{m}$$

$$\bar{v} = \sum_{i=1}^{n} \frac{v_i}{n}$$

$$GM_x = \exp(\bar{u})$$

$$GM_y = \exp(\bar{v})$$

The true percentage change of concentration (Δ) was calculated as follows:

$$S^2 = \frac{\sum_{i=1}^{m} \left(u_i - \bar{u}\right)^2 + \sum_{i=1}^{n} \left(v_i - \bar{v}\right)^2}{m + n - 2}$$

$$S_{\bar{v}-\bar{u}} = \sqrt{S2.\left(\frac{1}{m} + \frac{1}{n}\right)}$$

$$a = \bar{v} - \bar{u} - t.S_{\bar{v}-\bar{u}}$$

$$b = \bar{v} - \bar{u} + t.S_{\bar{v}-\bar{u}}$$

Where t is the 95th percentile of the $\underline{t}$ distribution with (m + n -2) degrees of freedom.

$$LL = (\exp(a) - 1).\ 100\ (\%)$$
$$UL = (\exp(b) - 1).\ 100\ (\%)$$

where LL and UL are the lower and upper limits of the 90% confidence interval for Δ .

TABLE A3.2 Statistical calculations for stability of mephenoxalone in spiked serum (10.0µg/ml, 24 hr, ambient temperature)

SAMPLE NUMBER	CONCENTRATION (µg/ml)	LN CONC
Freshly Prepared Samples		
1	10.95 (X_1)	2.393 (U_1)
2	10.81 (X_2)	2.380 (U_2)
3	10.84 (X_3)	2.383 (U_3)
4	10.92 (X_4)	2.391 (U_4)
5	10.75 (X_5)	2.375 (U_5)
		2.385 ($\overline{U}$)
Stored Samples		
1	10.78 (Y_1)	2.378 (V_1)
2	10.75 (Y_2)	2.375 (V_2)
3	10.82 (Y_3)	2.381 (V_3)
4	10.91 (Y_4)	2.390 (V_4)
5	10.85 (Y_5)	2.384 (V_5)
		2.382 ($\overline{V}$)

$GM_x = 10.86$; $GM_y = 10.83$; $s^2 = 4.39 \times 10^{-5}$; $S_{\overline{v}-\overline{u}} = 0.0118$;

df = 8; t 0 1.860; a = -0.0249; b = 0.0198; D = 0.29%;

LL = -2.46%; UL = 1.91%

TABLE A4.1 Dissolution results from powder in 3 dissolution media (USP paddle method)

DISSOLUTION MEDIUM	TIME (HOURS)	FRACTION OF POWDER (200mg) DISSOLVED			MEAN	(SD)
		1	2	3		
1000ml	0.00	0.000	0.000	0.000	0.000	(0.000)
WATER	0.17	0.616	0.517	0.621	0.585	(0.048)
	0.33	0.882	0.671	0.685	0.746	(0.096)
	0.50	0.961	0.759	0.802	0.841	(0.087)
	0.67	1.015	0.898	0.912	0.957	(0.059)
	0.83	1.030	1.001	1.013	1.015	(0.012)
	1.00	1.039	1.022	1.013	1.025	(0.011)
1000ml	0.00	0.000	0.000	0.000	0.000	(0.000)
	0.17	0.861	0.695	0.721	0.759	(0.073)
I.F.	0.33	0.976	0.851	0.897	0.908	(0.052)
	0.50	1.001	0.924	0.985	0.970	(0.033)
	0.67	1.002	0.998	0.992	0.997	(0.004)
	0.83	1.004	1.000	1.010	1.005	(0.004)
	1.00	1.004	1.004	1.017	1.008	(0.006)
1000ml	0.00	0.000	0.000	0.000	0.000	(0.000)
G.F.	0.17	0.661	0.584	0.512	0.586	(0.061)
	0.33	0.979	0.818	0.782	0.860	(0.086)
	0.50	1.006	0.898	0.895	0.933	(0.052)
	0.67	1.014	0.910	0.912	0.945	(0.049)
	0.83	1.021	0.942	0.989	0.984	(0.032)
	1.00	1.047	1.001	0.992	1.013	(0.024)

TABLE A4.2 Dissolution results from tablets in gastric fluid (USP paddle method)

TIME (H)	FRACTION (OF STATED AMOUNT) DISSOLVED FROM A SINGLE TABLE CONTAINING 200mg MEPHENOXALONE						MEAN	(SD)
	1	2	3	4	5	6		
0.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	(0.000)
0.17	0.910	0.874	0.942	0.790	0.850	0.931	0.833	(0.057)
0.33	0.930	0.889	0.948	0.900	0.926	0.938	0.922	(0.023)
0.50	0.942	0.930	0.953	0.953	0.929	0.941	0.941	(0.011)
0.67	0.945	0.934	0.956	0.955	0.942	0.970	0.950	(0.013)
0.83	0.956	0.941	0.960	0.957	0.951	0.972	0.956	(0.010)
1.00	0.958	0.949	0.971	0.961	0.967	0.974	0.963	(0.009)
1.17	0.968	0.954	0.976	0.963	0.969	0.980	0.968	(0.009)
1.33	0.976	0.963	0.978	0.973	0.975	0.983	0.975	(0.007)
1.50	0.977	0.964	0.979	0.973	0.980	0.984	0.976	(0.007)
1.67	0.989	0.979	0.988	0.977	0.980	0.996	0.985	(0.007)
1.83	1.022	0.989	1.003	0.983	0.989	0.998	0.997	(0.014)
2.00	1.026	1.002	1.026	0.988	0.989	1.000	1.005	(0.018)

TABLE A5.1 Mephenoxalone serum concentrations after the administration of mephenoxalone dispersion (Trial 1)

TIME (HOURS)	CONCENTRATION OF MEPHENOXALONE IN SERUM (µg/ml)						MEAN	(SD)
	WA	DW	MG	KZ	KR	KD		
0	0	0	0	0	0	0	0	(0)
0.17	0.49	2.55	0.40	0.45	0.37	0.64	0.82	(0.78)
0.33	1.04	3.91	1.77	1.67	2.52	3.25	2.36	(0.98)
0.50	4.52	4.33	3.75	2.56	3.74	5.49	4.07	(0.89)
0.67	8.85	5.60	6.20	3.63	7.30	8.21	6.63	(1.74)
0.83	6.27	6.46	6.61	4.35	7.40	10.22	6.89	(1.75)
1.00	5.70	6.24	7.66	5.98	7.65	11.42	7.44	(1.94)
1.17	5.66	4.51	7.00	7.41	7.48	11.31	7.23	(2.11)
1.33	5.04	4.32	6.30	6.97	7.33	10.80	6.79	(2.07)
1.50	4.75	4.02	6.02	6.68	6.99	10.70	6.53	(2.14)
1.75	4.51	3.99	5.68	6.30	6.95	10.15	6.26	(2.01)
2.00	4.28	3.57	5.39	5.95	6.67	9.80	5.94	(2.00)
2.50	3.55	3.05	4.60	5.50	6.45	8.45	5.27	(1.82)
3.00	3.05	2.50	3.95	4.73	5.95	7.99	4.70	(1.85)
4.00	2.10	1.84	2.89	3.33	5.26	6.25	3.61	(1.62)
5.00	1.70	1.31	2.07	2.72	4.75	5.06	2.94	(1.46)
6.00	1.18	0.89	1.48	1.88	4.25	3.89	2.26	(1.32)
8.00	0.64	0.51	0.78	1.20	2.99	2.70	1.47	(1.00)
10.00	0.34	0.30	0.45	0.91	2.15	1.60	0.96	(0.70)
12.00	0.18	0.15	0.24	0.37	1.52	1.02	0.58	(0.51)
24.00	0	0	0	0	0.36	0	0.06	(0.13)

TABLE A5.2 Mephenoxalone serum concentrations after the administration of tablets (Trial 2)

TIME (HOURS)	CONCENTRATION OF MEPHENOXALONE IN SERUM (µg/ml)					MEAN	(SD)
	WA	DW	MG	KZ	KR		
0	0	0	0	0	0	0	(0)
0.17	3.13	0.78	0	0	0.15	0.81	(1.19)
0.33	12.14	9.45	0.36	0.19	3.17	5.06	(4.87)
0.50	9.83	7.77	0.91	0.49	6.06	5.01	(3.72)
0.67	8.69	6.70	0.90	2.51	7.24	5.21	(2.98)
0.83	7.68	6.00	1.00	14.39	11.00	8.01	(4.54)
1.00	7.42	5.73	1.19	11.93	9.75	7.20	(3.67)
1.17	6.91	5.50	1.01	10.72	9.52	6.73	(3.40)
1.33	6.55	5.21	1.28	NS	9.38	5.61	(2.92)
1.50	6.33	4.98	3.22	10.09	9.28	6.78	(2.58)
1.75	5.71	4.49	4.76	9.40	9.15	6.70	(2.14)
2.00	5.47	4.39	5.39	9.08	8.59	6.58	(1.88)
2.50	4.67	3.53	6.10	8.61	7.84	6.15	(1.90)
3.00	3.94	2.81	5.26	7.85	6.73	5.32	(1.82)
4.00	2.73	2.42	3.84	6.74	5.75	4.30	(1.69)
5.00	2.10	1.96	3.09	5.70	4.93	3.56	(1.51)
6.00	1.39	1.39	1.90	4.23	3.81	2.54	(1.23)
8.00	0.89	0.71	1.12	2.80	2.60	1.62	(0.89)
10.00	0.56	0.41	0.67	1.93	1.65	1.04	(0.62)
12.00	0.32	0.20	0.30	1.23	1.09	0.63	(0.44)
24.00	0	0	0	0.19	0.19	0.08	(0.09)

NS = no sample drawn

TABLE A5.3 The cumulative urinary excretion of mephenoxalone after the administration of 400mg mephenoxalone powder dispersed in 150ml water

TIME INTERVAL (HOURS)	CUMULATIVE AMOUNT OF MEPHENOXALONE EXCRETED IN URINE (MG)						MEAN	(SD)
	WA	DW	MG	KZ	KR	KD		
0	0	0	0	0	0	0	0	(0)
0 to 2	3.91	2.26	1.22	4.54	2.52	1.24	2.62	(1.25)
2 to 4	4.50	2.82	1.81	6.87	5.34	1.85	3.87	(1.87)
4 to 6	4.69	3.00	2.02	7.25	6.19	2.07	4.20	(2.01)
6 to 10	4.92	3.00	2.31	7.55	7.08	2.33	4.53	(2.16)
10 to 14	4.92	3.00	2.31	7.55	7.90	2.33	4.67	(2.33)
14 to 24	4.92	3.00	2.31	7.55	7.90	2.33	4.67	(2.33)

TABLE A5.4 Observed and model-predicted serum concentrations of mephenoxalone (using model A) following administration of a 400mg dispersed dose of the drug.

TIME (HOURS)	WA		DW		MG		K2		KR		KD		MEAN DATA	
	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.17	0.49	2.74	2.55	3.19	0.40	2.05	0.45	1.99	0.37	2.36	0.64	2.96	0.82	2.16
0.33	1.04	3.62	3.91	4.05	1.77	3.48	1.67	2.68	2.52	3.53	3.25	5.12	2.36	3.66
0.50	4.52	4.54	4.33	4.81	3.75	4.58	2.56	3.88	3.74	4.99	5.49	6.87	4.07	4.81
0.67	8.85	5.07	5.60	5.13	6.20	5.34	3.63	4.38	7.30	5.96	8.21	8.16	6.63	5.62
0.83	6.27	5.32	6.46	5.19	6.61	5.82	4.35	4.92	7.40	6.28	10.22	9.04	6.89	6.13
1.00	5.70	5.42	6.24	5.11	7.66	6.13	5.98	5.34	7.65	6.67	11.42	9.70	7.44	6.47
1.17	5.66	5.40	4.51	4.95	7.00	6.28	7.41	5.63	7.48	6.91	11.31	10.12	7.23	6.66
1.33	5.04	5.31	4.32	4.75	6.30	6.31	6.97	5.82	7.33	7.08	10.80	10.35	6.79	6.73
1.50	4.75	5.16	4.02	4.53	6.02	6.26	6.68	5.94	6.99	7.19	10.70	10.45	6.53	6.71
1.75	4.51	4.89	3.99	4.19	5.68	6.06	6.30	5.99	6.95	7.22	10.15	10.40	6.26	6.57
2.00	4.28	4.59	3.57	3.86	5.39	5.77	5.95	5.94	6.67	7.15	9.80	10.18	5.94	6.34
2.50	3.55	3.99	3.05	3.25	4.60	5.06	5.50	5.64	6.45	6.97	8.45	9.43	5.27	5.74
3.00	3.05	3.43	2.50	2.73	3.95	4.32	4.73	5.19	5.95	6.40	7.99	8.51	4.70	5.09
4.00	2.10	2.44	1.84	1.91	2.89	3.02	3.33	4.12	5.26	5.49	6.25	6.65	3.61	3.89
5.00	1.70	1.84	1.31	1.36	2.07	2.05	2.72	3.29	4.75	4.71	5.06	5.08	2.94	2.93
6.00	1.18	1.31	0.89	0.93	1.48	1.39	1.88	2.54	4.25	3.95	3.89	3.84	2.26	2.20
8.00	0.64	0.70	0.51	0.45	0.78	0.62	1.20	1.50	2.99	2.88	2.70	2.18	1.47	1.23
10.00	0.34	0.38	0.30	0.24	0.45	0.28	0.91	0.88	2.15	2.07	1.60	1.23	0.96	0.69
12.00	0.18	0.20	0.15	0.12	0.24	0.13	0.37	0.51	1.52	1.49	1.02	0.69	0.58	0.39
24.00	0	0	0	0	0	0	0	0	0.36	0.21	0	0	0.06	0

TABLE A5.5 Observed and model-predicted serum concentrations of mephenoxalone (using model A) following administration of a 400mg tablet dose of the drug.

TIME (HOURS)	WA		DW		MG		K2		KR		MEAN DATA	
	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.
0	0	0	0	0	0	0	0	0	0	0	0	0
0.17	3.13	6.68	0.78	4.42	0	0.64	0	2.90	0.15	3.44	0.81	2.29
0.33	12.14	8.02	9.45	6.07	0.36	1.17	0.19	4.05	3.17	4.79	5.06	3.87
0.50	9.83	8.50	7.77	6.63	0.91	1.66	0.49	5.51	6.06	6.35	5.01	5.07
0.67	8.69	8.39	6.70	6.66	0.90	2.08	2.51	6.65	7.24	7.58	5.21	5.90
0.83	7.68	8.11	6.00	6.41	1.00	2.42	14.39	7.48	11.00	8.19	8.01	6.42
1.00	7.42	7.75	5.73	6.12	1.19	2.73	11.93	8.14	9.75	8.65	7.20	6.76
1.17	6.91	7.38	5.50	5.86	1.01	2.99	10.72	8.80	9.52	8.97	6.73	6.95
1.33	6.55	7.04	5.21	5.56	1.28	3.20	NS	-	9.38	9.18	5.61	7.01
1.50	6.33	6.63	4.98	5.30	3.22	3.37	10.09	9.18	9.28	9.25	6.78	6.99
1.75	5.71	6.21	4.49	4.81	4.76	3.57	9.40	9.34	9.15	9.22	6.70	6.84
2.00	5.47	5.73	4.39	4.36	5.39	3.71	9.08	9.35	8.59	9.03	6.58	6.61
2.50	4.67	4.96	3.53	3.70	6.10	3.82	8.61	9.09	7.84	8.41	6.15	6.01
3.00	3.94	4.18	2.81	3.11	5.26	3.79	7.85	8.60	6.73	7.65	5.32	5.36
4.00	2.73	3.16	2.42	2.19	3.84	3.45	6.74	7.25	5.75	6.11	4.30	4.17
5.00	2.10	2.40	1.96	1.61	3.09	3.08	5.70	6.52	4.93	4.88	3.56	3.20
6.00	1.39	1.63	1.39	1.03	1.90	2.45	4.23	5.05	3.81	3.57	2.54	2.45
8.00	0.89	0.93	0.71	0.53	1.12	1.52	2.80	3.61	2.60	2.25	1.62	1.44
10.00	0.56	0.52	0.41	0.27	0.67	0.95	1.93	2.50	1.65	1.35	1.04	0.84
12.00	0.32	0.29	0.20	0.13	0.30	0.56	1.23	1.72	1.09	0.81	0.63	0.49
24.00	0	0	0	0	0	0	0.19	0.19	0.19	0.04	0.08	0

NS = no sample withdrawn

TABLE A5.6 Observed and model-predicted serum concentrations of mephenoxalone (using model B) following administration of a 400mg dispersed dose of the drug (Trial 1).

TIME (HOURS)	WA		DW		MG		KZ		KR		KD		MEAN DATA	
	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.17	0.49	2.63	2.55	3.08	0.40	2.05	0.45	1.92	0.37	2.34	0.64	2.88	0.82	2.06
0.33	1.04	3.52	3.91	3.99	1.77	3.47	1.67	2.60	2.52	3.50	3.25	4.99	2.36	3.53
0.50	4.52	4.53	4.33	4.83	3,75	4.58	2.56	3.82	3.74	4.97	5.49	6.70	4.07	4.71
0.67	8.85	5.15	5.60	5.22	6.20	5.34	3.63	4.34	7.30	5.96	8.21	7.97	6.63	5.56
0.83	6.27	5.47	6.46	5.31	6.61	5.81	4.35	4.90	7.40	6.29	10.22	8.84	6.89	6.12
1.00	5.70	5.62	6.24	5.23	7.66	6.11	5.98	5.35	7.65	6.69	11.42	9.48	7.44	6.50
1.17	5.66	5.61	4.51	5.04	7.00	6.25	7.41	5.67	7.48	6.93	11.31	9.89	7.23	6.72
1.33	5.04	5.51	4.32	4.81	6.30	6.27	6.97	5.87	7.33	7.10	10.80	10.11	6.79	6.81
1.50	4.75	5.33	4.02	4.53	6.02	6.20	6.68	6.01	6.99	7.20	10.70	10.20	6.53	6.80
1.75	4.51	4.99	3.99	4.10	5.68	5.98	6.30	6.07	6.95	7.23	10.15	10.12	6.26	6.65
2.00	4.28	4.50	3.57	3.69	5.39	5.67	5.95	6.01	6.67	7.14	9.80	9.88	5.94	6.39
2.50	3.55	3.80	3.05	2.97	4.60	4.93	5.50	5.65	6.45	6.93	8.45	9.09	5.27	5.69
3.00	3.05	3.09	2.50	2.40	3.95	4.17	4.73	5.10	5.95	6.30	7.99	8.13	4.70	4.93
4.00	2.10	1.96	1.84	1.62	2.89	2.88	3.33	3.80	5.26	5.33	6.25	6.28	3.61	3.58
5.00	1.70	1.40	1.31	1.18	2.07	1.98	2.72	2.82	4.75	4.53	5.06	4.78	2.94	2.59
6.00	1.18	1.00	0.89	0.90	1.48	1.39	1.88	2.00	4.25	3.78	3.89	3.69	2.26	1.94
8.00	0.64	0.66	0.51	0.64	0.78	0.77	1.20	1.01	2.99	2.81	2.70	2.35	1.47	1.23
10.00	0.34	0.54	0.30	0.53	0.45	0.51	0.91	0.57	2.15	2.13	1.60	1.67	0.96	0.92
12.00	0.18	0.47	0.15	0.45	0.24	0.40	0.37	0.38	1.52	1.67	1.02	1.31	0.58	0.75
24.00	0	0	0	0	0	0	0	0	0.36	0.59	0	0	0.06	0.21

TABLE A5.7 Observed and model-predicted serum concentrations of mephenoxalone (using model B) following administration of a 400mg tablet dose of the drug (Trial 2).

TIME (HOURS)	WA		DW		MG		KZ		KR		MEAN DATA	
	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.	OBS.	PRED.
0	0	0	0	0	0	0	0	0	0	0	0	0
0.17	3.13	6.66	0.78	4.20	0	0.59	0	2.72	0.15	3.54	0.81	2.20
0.33	12.14	8.25	9.45	6.04	0.36	1.08	0.19	3.84	3.17	4.92	5.06	3.80
0.50	9.83	8.91	7.77	6.78	0.91	1.53	0.49	5.32	6.06	6.51	5.01	5.04
0.67	8.69	8.79	6.70	6.87	0.90	1.93	2.51	6.52	7.24	7.75	5.21	5.87
0.83	7.68	8.42	6.00	6.58	1.00	2.25	14.39	7.43	11.00	8.35	8.01	6.39
1.00	7.42	7.91	5.73	6.22	1.19	2.54	11.93	8.19	9.75	8.80	7.20	6.74
1.17	6.91	7.38	5.50	5.87	1.01	2.79	10.72	8.99	9.52	9.09	6.73	6.93
1.33	6.55	6.90	5.21	6.04	1.28	2.98	NS	-	9.38	9.28	5.61	6.99
1.50	6.33	6.34	4.98	5.14	3.22	3.16	10.09	9.48	9.28	9.32	6.78	6.97
1.75	5.71	5.79	4.49	4.53	4.76	3.35	9.40	9.73	9.15	9.24	6.70	6.82
2.00	5.47	5.18	4.39	4.03	5.39	3.49	9.08	9.79	8.59	8.98	6.58	6.58
2.50	4.67	4.30	3.53	3.36	6.10	3.61	8.61	9.52	7.84	8.27	6.15	5.96
3.00	3.94	3.50	2.81	2.86	5.26	3.59	7.85	8.91	6.73	7.43	5.32	5.30
4.00	2.73	2.61	2.42	2.12	3.84	3.30	6.74	7.12	5.75	5.82	4.30	4.09
5.00	2.10	2.05	1.96	1.87	3.09	2.96	5.70	6.14	4.93	4.61	3.56	3.14
6.00	1.39	1.57	1.39	1.54	1.90	2.36	4.23	4.26	3.81	3.40	2.54	2.41
8.00	0.89	1.18	0.71	1.21	1.12	1.48	2.80	2.71	2.60	2.29	1.62	1.45
10.00	0.56	0.95	0.41	0.96	0.67	0.93	1.93	1.78	1.65	1.63	1.04	0.89
12.00	0.32	0.78	0.20	0.76	0.30	0.56	1.23	1.31	1.09	1.26	0.63	0.56
24.00	0	0	0	0	0	0	0.19	0.76	0.19	0.66	0.08	0.18

NS = no sample withdrawn

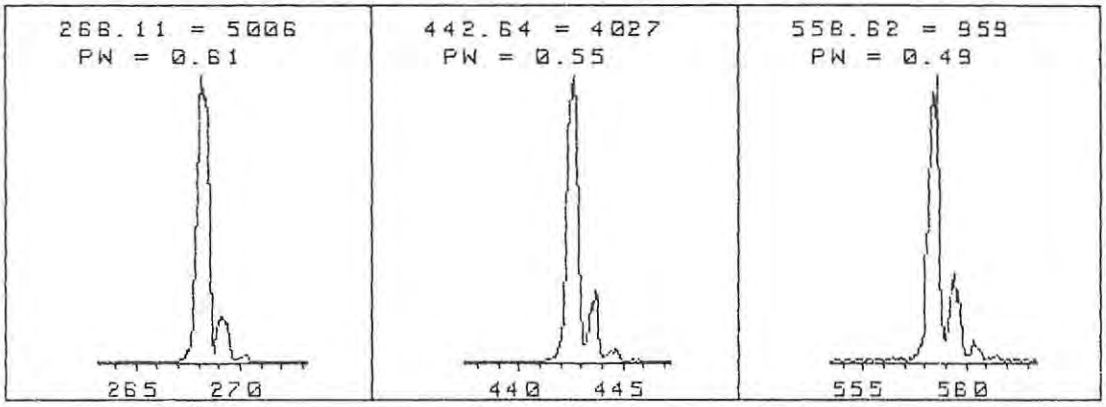
TABLE A5.8 Parameter estimates from computer modelling of dispersion and tablet dosage data

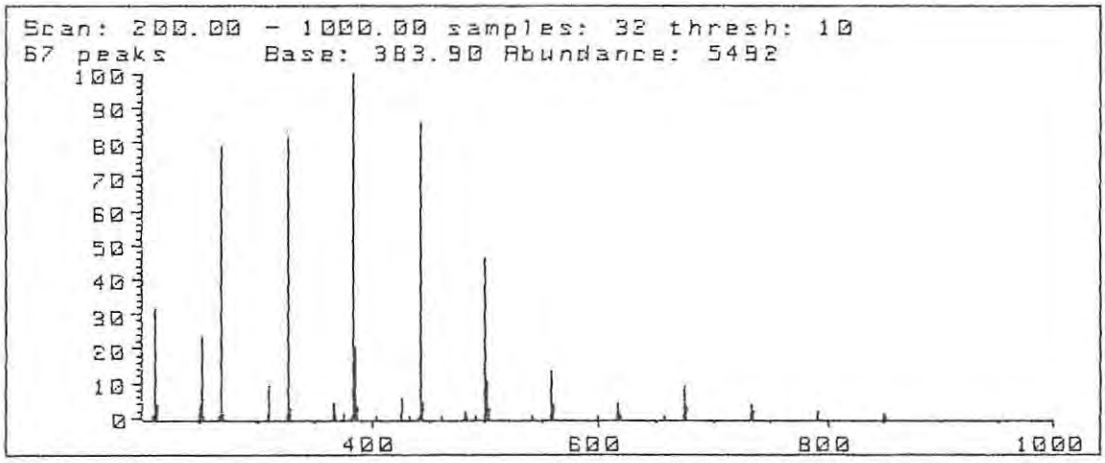
MODEL	PARAMETER	SUBJECTS						PARAMETER ESTIMATES FROM MEAN DATA
		WA	DW	MG	KZ	KR	KD	
400mg DISPERSION STUDY								
1BCM	ka	2.152	3.000	1.307	1.053	1.452	1.211	1.471
1ka	V_1	64.085	83.718	66.284	51.539	56.119	51.207	68.643
linear	k_{10}	0.314	0.350	0.400	0.268	0.165	0.286	0.289
elimination	tlag	0.879	0.122	0.434	0.198	0.201	0.610	0.368
	r[a]	0.879	0.970	0.957	0.953	0.959	0.969	0.969
2BCM	ka	1.475	2.314	1.231	0.679	1.347	1.098	1.045
1ka	V_1	61.888	82.383	65.680	46.071	55.490	50.547	61.190
linear	k_{12}	0.247	0.229	0.155	0.225	0.609	0.135	0.193
elimination	k_{21}	0.086	0.131	0.583	0.022	0.116	0.086	0.116
	k_{10}	0.312	0.314	0.312	0.264	0.147	0.224	0.298
	tlag	0.304	0.299	0.501	0.712	0.252	0.720	0.671
	r	0.855	0.974	0.960	0.955	0.917	0.973	0.971
400mg TABLET STUDY								
1BCM	ka	5.930	4.777	0.500	1.145	1.295	-	1.551
1ka	V_1	76.303	87.900	56.068	54.484	54.227	-	67.068
linear	k_{10}	0.300	0.350	0.286	0.185	0.254	-	0.268
elimination	tlag	0.1083	0.124	0.248	0.519	0.483	-	0.341
	r	0.925	0.899	0.833	0.834	0.953	-	0.961
2BCM	ka	4.708	3.459	0.436	0.695	1.252	-	1.484
1ka	V_1	61.530	85.672	54.440	55.111	53.811	-	67.687
linear	K_{12}	0.203	0.348	0.128	0.191	0.132	-	0.026
elimination	K_{21}	0.174	0.334	0.973	0.039	0.058	-	0.300
	k_{10}	0.280	0.297	0.309	0.180	0.177	-	0.272
	tlag	0.122	0.221	0.333	1.457	0.543	-	0.383
	r	0.930	0.904	0.840	0.836	0.907	-	0.961

a. r = correlation coefficient

Table A6.1 TSP tune report

```
M A N U A L   T U N E     TXTUN1.U
Misc Info   :  PolyPropylene Glycol (PPG)
Instrument  :  Thermo Spray 5888 Extended Mass
Multiplier   1724    Integration   150     Emission      ON
  RMU gain    156     RMU offset   207     Repeller    7.70
 Ion focus  195.0     Entr. lens   165        X ray    80.0
 Axis gain   -133    Axis offset    42     Draw Out    55.0
   Samples     32       Averages     1     Stepsize    0.10
```





Mass	Abund	Rel Abund	Iso Mass	Iso Abund	Iso Ratio
268.05	4328	100.00	269.05	628	14.51
442.50	4690	108.36	443.50	1039	22.15
558.25	746	17.24	559.25	211	28.28

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